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in

Fundamentals of
BACTERIOLOGY

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CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE, * MYSORE. *
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Fourth Edition, Illustrated

W. B. SAUNDERS COMPANY
Philadelphia and London
1950

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Reprinted October, 1949, and June, 1950

MADE IN U. S. A.

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PHILADELPHIA

PREFACE TO THE FOURTH EDITION

The principles which guided previous revisions of this textbook have been followed in preparing this, the fourth edition. As in previous editions the approach is functional and the viewpoint practical. The style of presentation is for beginning students with a moderate knowledge of elementary biology, physics and chemistry, including some organic chemistry.

No effort has been spared to bring the new edition into line with the latest advances in the field of microbiology. A very extensive literature, most of it published since the last edition, has been reviewed and pertinent material for each chapter has been carefully selected, digested, and incorporated into the new book. Such material is found on almost every page. For example, the latest views on cell structure, new methods in electron and phase microscopy and new theories of enzyme action are discussed as well as recent ideas on bacterial variation and mutation, sexes in bacteria, mutagenic agents, viruses and their relation to cancer, disinfectants, and so on through a long list of topics.

There is a complete new chapter on the Microbiology of Food, with discussion of domestic applications related to food preservation and food spoilage. The material on Soil Microbiology has been made more complete. New ideas of petroleum microbiology are outlined. Industrial microbiology has interesting new material such as the Madison wood sugar process, the modified amylo process and an illustrative procedure for making cheese in the home. The chapter on Microbiology of Air is enlarged and modernized and includes new studies on air examination and air disinfection. The chapters on viruses are much more complete, with new information on German measles, poliomyelitis, the common cold, etc. It would require more space than is appropriate in a preface to outline all of the changes, deletions of old material and inclusion of new data and ideas which may be found in this book. Many old illustrations have been discarded and new and better ones included.

The new edition is divided into four sections: first, Basic Principles, dealing with history, ecology, structure, metabolism, disinfection, etc.; second, The Yeasts, Molds and Higher Bacteria, including Actinomycetales, Spirochaetales, etc.; third, The True Bacteria, including agricultural, industrial, economic, domestic and related phases of bacteriology; fourth, The Pathogenic Microorganisms, including Protozoa, and the diseases caused by each. The first three sections are intended to serve in a one or two semester (or trimester) introductory cours The relation of microorganisms to disease (except for certain bri incidental references) is outlined in only two general chapters. Th fourth section is intended for later courses in pathogenic microbiolog but the material may be included as supplementary reading to an introductory course, however brief. This is because a style of presentation is maintained throughout which makes any of the subjects, fro first to last chapter, clear to the intelligent and fairly educated reade whether his interest lies in learning what bacteria are, their natura history and general properties; in electron and phase microscopy; i Lamarckism versus Darwinism in microbiology; in new methods o using and producing enzymes industrially; the role of bacteria in so fertility or food spoilage; the reality of flagella; German measles as cause of fetal defects during pregnancy; or in the relation of pleuropneumonia organisms to protozoa, rickettsiae or algae. The book i obviously larger and more widely inclusive than previously but it is no more complex or abstruse in style of presentation.

As in the previous edition, the author has had the kind cooperatio of Dr. Marion Brooke, Chief, Parasitology Laboratories, Communicable Disease Center, U. S. Public Health Service, in preparing th new chapter on protozoa. This adds greatly to its value. Gratefu acknowledgment is made also of the untiring and painstaking assistanc of Hermine G. Bird, Editorial Assistant of the "American Journal o Hygiene," in early preparation of the manuscript and in arranging an compiling references. Valuable aid with references was also rendere by Miss Nelle Barmore, Librarian of the Communicable Diseas Center in Atlanta. To the lot of the long-suffering but always cheerfu wife of the author fell the un-inspiring role of assistant proofreader index maker and general helper and encourager; a true genius of thi work. The editorial staff and all concerned at the publishers' end o the project deserve a round of applause and medals for producing fine craftsman's product from very difficult raw material. Sincere thank are given to the many authors, colleagues, and publishers who so generously granted permission for the use of illustrative matter, especially t the Committee on Visual Materials for Visual Instruction in Microbiology (H. H. Morton, Chairman) for original electron microscop photographs and other unique materials obtained from distinguishe investigators all over the country; and to the many readers and teachers who took the time and trouble to convey their criticisms and suggestions to the author or publisher for improvement. The author hopes that many of them will recognize in the text the impact of their ideas.

Atlanta, Georgia

MARTIN FROBISHER, JR. SC. D.

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Section I

FIRST PRINCIPLES

Chapter 1

INTRODUCTION

The Origin and Discovery of Bacteria. The visual experience of those to whom the microscope is unknown fails to encompass any living thing smaller than insects like fleas, lice and mites, or some of the algae. The bacteriologist, however, has entrée into an amazing universe where those ignorant of the subject cannot follow. He becomes familiar with hordes of beautiful creatures, visible only beneath his lenses and, by special means, also becomes aware of the existence of mysterious, physiologically active structures so small as to be invisible even with the most powerful ordinary microscope but of immense importance industrially and medically.

Although bacteria are extremely minute, measuring usually about 1/50,000 of an inch in diameter and weighing as little as 4/10,000,000,000,000 (four ten trillionths) of a gram, it must not be thought that on this account they are unimportant or that they experience undue difficulty in the struggle for existence. Survival and persistence of a species do not depend on great size alone, but upon the power to live and multiply under a variety of conditions. Let us imagine creatures, for example, which can function in a range of temperatures from freezing to almost boiling, and regardless of whether free oxygen and food, as we know it, be present or not; creatures which, in addition to actively carrying on the business of life under these circumstances, can double their numbers every twenty minutes or, if required, cease growth and go into a state of what seems to be completely suspended animation, in this state surviving cold so intense as to liquefy hydrogen, extreme drought prolonged for many years, heat so intense as to coagulate blood, high pressures and high vacua. Let us imagine, furthermore, that the creatures are so minute as to be invisible to any enemies possessed of eyes with which to hunt them. Such creatures, it is clear, would be far more likely to survive the rigors of environmental change, and competition with larger forms of life, than unwieldy and relatively vulnerable structures like men, animals and trees. Such gifted organisms are the bacteria and similar microorganisms. Some of them are admirably adapted to survive under conditions such as those described, some of which probably existed in the early geological history of the earth;[1] and to survive all the known vicissitudes of climatic change since those periods to the exclusion, if need be, of other, more advanced forms, of life. Not all species of bacteria possess all of the properties enumerated, but some of them do and these are among the most interesting creatures known. It seems not unlikely that, unless some grand cataclysm destroy all living things suddenly, such bacteria may be the last survivors of the pageant of life on this globe.

The Primitive World. Bacteria are very primitive structures and it may therefore be supposed that they represent the type of living beings which appeared very early in the earth's history of life. Indeed, for many years bacteria were regarded as probably the first cellular inhabitants of this planet.

The primitive bacteria of primeval times probably lived in the sea or mud, and from them probably evolved the saprophytic and pathogenic bacteria familiar to us today.[2, 3, 4] Familiarity with bacteria, however, is of rather recent achievement since they could be studied accurately only after the development of lenses of sufficient magnifying power to make them visible. The role of the microscope in bacteriology, therefore, is important.

The First Microscopes. Leeuwenhoek. Use of lenses has thrown much light upon all the questions which occupied the earliest biologists. By the end of the seventeenth century lenses had already been exploited in various ways for many years, Roger Bacon having used them experimentally before 1294. Such lenses, however, did not magnify very highly. The man mainly responsible for revealing the whole, hitherto unknown and unseen world of bacteria and other minute plants and animals, was the Dutch investigator (Fig. 1), *Antonj van Leeuwenhoek* (1632–1723), a linen merchant by trade and a successful politician as well. Leeuwenhoek was an active, intelligent man of public and commercial affairs in the city of Delft. He was not a trained scientist but was self-educated. He amused himself by means of his skill and craftsmanship in glass blowing, fine metal work and other occupations. He came of a well-to-do and influential family and lived in relatively easy circumstances with plenty of leisure for his avocation of making minute lenses. With these he delighted in examining a great variety of objects. He examined saliva, pepper decoctions, cork, the leaves of plants, circulating blood in the tail of a salamander, seminal fluid, urine, cowdung, scrapings from the teeth and so on. In many of these he saw living creatures which we now know were bacteria but which he called "animalcules." ("Animalcule" is a term which was used by the earlier workers to designate any minute plant or animal.)

His interest lay chiefly in his microscopes, however, and only secondarily in what he saw with them, although the latter fascinated him. In spite of the fact that his microscopes were not compound he obtained remarkable results with them. ". . . he showed rare ingenuity and expert craftsmanship in the grinding and mounting of his simple lenses, a skill which he zealously kept to himself; and in spite of the requests of his learned friends, he refused to disclose the secret of his success." ". . . Leeuwenhoek's instruments are not true microscopes at all in the sense in which we think of microscopes, but rather simple magnifying glasses generally consisting of a small, single, biconvex lens. The object, and not the lens, was moved into focus by means of screws" (Fig. 2). "To adjust the lens to the object was so long and tedious a task that it is not surprising that Leeuwenhoek used an individual lens for each object. . . ." "The magnification varied and at best did not exceed two hundred to three hundred diameters." "The size of objects which Leeuwenhoek examined was determined by comparison. For this purpose he used at various times a grain of sand, the seed of millet or mustard, the eye of a louse, a vinegar eel, and still later hair or blood corpuscles. In this way he secured fairly accurate measurements of a great variety of objects." ". . . he was forced to admit that the sand grain was more than one million times the size of one of the animalcules."[5]

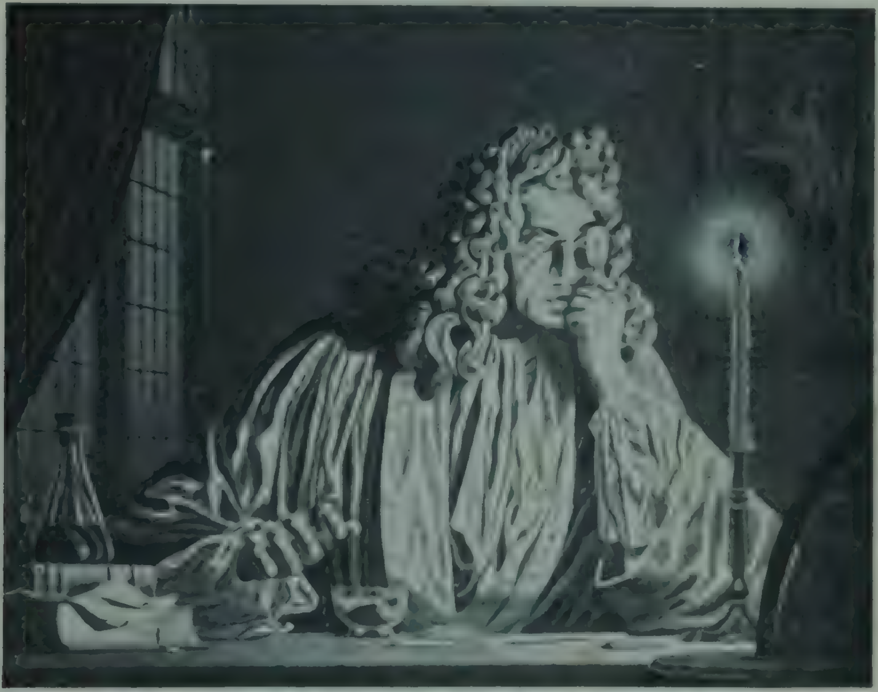

Fig. 1. Antonj van Leeuwenhoek. A fanciful delineation based on a famous portrait. The picture shows accurately the size and shape of the first microscopes, the manner in which they were used, and the simple laboratory apparatus of the "Father of Bacteriology." (Courtesy of Lambert Pharmacal Co.)

Leeuwenhoek was so charmed with the things he observed that he felt constrained to write minutely detailed reports about them to the

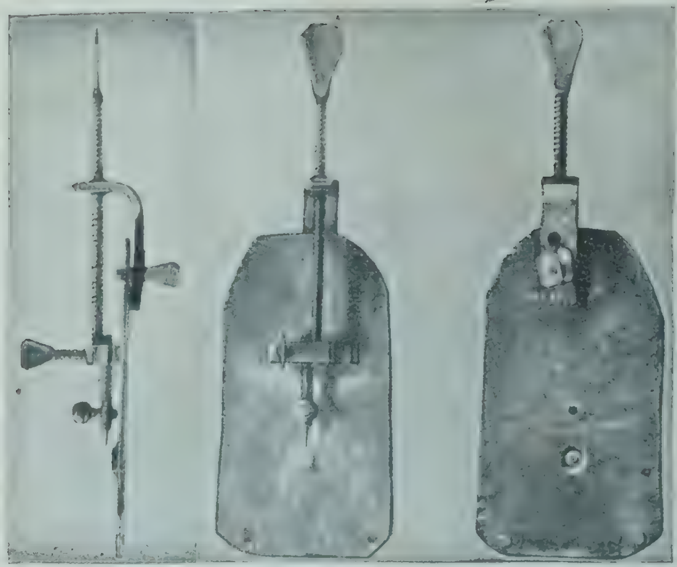

Fig. 2. One of Leeuwenhoek's microscopes: side, back and front views.

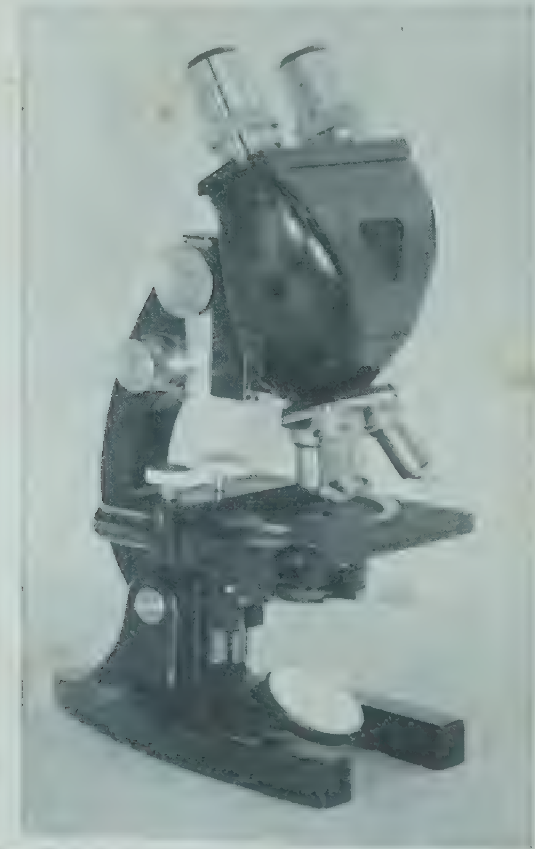

Fig. 3. Modern laboratory microscope. (Bausch & Lomb Optical Co.)

Royal Society in London[6] and was later elected a Fellow of that body. Some of his observations are at once quaint and epoch-making. For example, after examining material which he scraped from between his teeth, he said, "Though my teeth are kept usually very clean, nevertheless when I view them in a Magnifying Glass, I find growing between them a little white matter as thick as wetted flour; in this substance, though I could not perceive any motion, I judged there might probably be living Creatures.

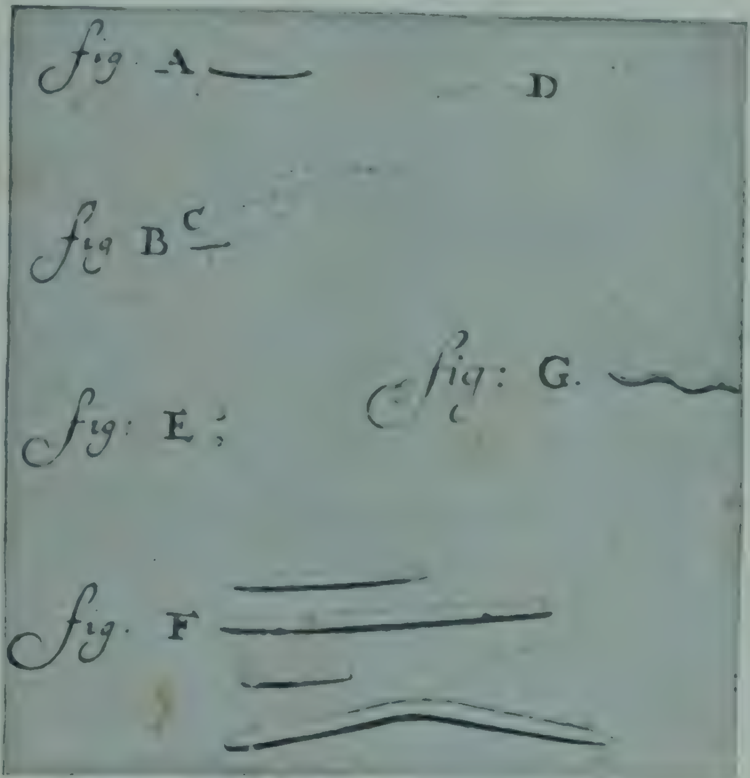

Fig. 4. Leeuwenhoek's drawings of bacteria.

"I therefore took some of this flour and mixt it either with pure rain water wherein were no Animals; or else with some of my Spittle (having no Air bubbles to cause a motion in it) and then to my great surprise perceived that the aforesaid matter contain very small living animals, which moved themselves very extravagantly. The biggest sort had the shape of A (Fig. 4). Their motion was strong and nimble, and they darted themselves through the water or spittle, as a Jack or Pike does through the water. These were generally not many in number. The second sort had the shape of B. These spun about like a top, or took a course sometimes on one side, as is shown at C and D. They were more in number than the first. In the third sort I could not well

distinguish the Figure, for sometimes it seem'd to be an Oval, ar
other times a Circle. These were so small they seem'd no bigger than
and therewithal so swift, that I can compare them to nothing bett
than a swarm of Flies or Gnats, flying and turning among one anoth
in a small space."

Natural Philosophy. Most of the men of Leeuwenhoek's time wl
were interested in problems of biology also interested themselves
questions concerning the origin of life. They were called students
Natural Philosophy. This included Zoology, Chemistry, Music, Astro
omy, Geography, Art, Mathematics, Medicine, Literature, and so o
Medical treatises of that day were sometimes written in poetry ar
illustrated with fanciful pictures. When bacteria, through the agency
microscopes, were first brought within the range of human vision, th
were viewed by students of natural philosophy from various stan
points. Some considered them the original and lowest forms of lif
others hailed them as the cause of disease. Furious debates arose, son
of which still rage on. Those concerning the source of bacteria and the
relation to the problem of the origin of life were especially vigoro
and make interesting reading. It is desirable to review some of the
ideas since a number of discoveries were made, during the studies
the subject, which are still the basis of present-day bacteriology. Wit
out a knowledge of these earlier discoveries, the student of bacteriolo
works at a disadvantage.

**Ancient Theories Concerning the Spontaneous Formation of Plar
and Animals.** The ancients knew nothing of bacteria and such prim
tive forms of life but believed that creatures like frogs, mice, bees ar
other animals sprang fully-formed from fertile mud, decaying ca
casses, warm rain or fog, and the like.

Van Helmont (1577–1644) devised a method for manufacturi
mice. He recommended putting some wheat grains with soiled lin
and cheese into an appropriate receptacle. Mice would then appe
This observation may still be *experimentally confirmed* but the co
clusions drawn from it differ today. However, an elderly lady of t
writer's acquaintance complained bitterly that she had been cheat
by a merchant who sold her a woolen coat which was of such a qual
that it turned entirely into moths when left undisturbed in a clo
for some months!

Such empirical theories of spontaneous generation of living bein
were later discarded; but only after a most dramatic intellectual a
experimental struggle, which will be detailed presently. All such th
ories assumed that life begins in an already highly developed sta
It was later realized that life must have originated in a much si
pler form.

After the development of the microscope an idea which held sw for some time was that life had its origin in the form of unicellular organisms usually thought of as having an amoeba-like structure and physiology. When one stops to consider the very complex nature of an amoeba, it is amazing that such an idea could have persisted so long. According to Minchin, "It is impossible any longer to regard the cell, as seen in metazoa and as defined in the text books, as the starting point of organic evolution. It must be recognized that this type of cell has a long history of evolution behind it."[4] If we are to believe that life began according to natural laws we must look for something simpler than the cell from which more complicated creatures evolved.

The Dispute Over Spontaneous Generation. In the earlier years, in the absence of exact knowledge of bacteria or chemistry and while Leeuwenhoek was amusing himself with lens making, there had already arisen much skepticism and bitter feeling over the question of the origin of life. One scientist who still held to the ancient ideas says of the views of another who doubted, "So may we doubt whether, in cheese and timber, worms are generated, or if beetles and wasps in cow dung, or if butterflies, locusts, shell-fish, snails, eels, and such life be procreated of putrefied matter which is to receive the forms of that creature to which it is by formative power disposed. To question this is to question reason, sense and experience. If he doubts this let him go to Egypt and there he will find the fields swarming with mice begot of the mud of Nylus, to the great calamity of the inhabitants."[7] There was a great deal of such acrid discussion by wordy savants of the times who tried to settle everything by argument. Experimentation was regarded as rather undignified and even smacking of relations with the evil one.*

Francesco Redi (1626–1679). The experimental method was, however, being invoked here and there. For example, it had always been supposed that the maggots in decaying meat were derived spontaneously from transformations of the putrid meat itself. Redi (Fig. 5), a poet and physician of Arezzo, placed meat and fish in jars covered with very fine gauze. He saw flies approach the jars and crawl on the gauze. He saw the eggs of the flies caught on the gauze and observed that the meat then putrefied without maggot formation. Maggots developed only when the flies' eggs were deposited on the meat itself. Redi's work was not widely noted, however, and it was not until much later that another series of experiments was made.

---

* The student will note three main phases of the question of spontaneous generation:

(*a*) That concerning the origin of life through chemical evolution early in the history of the planet.

(*b*) That concerning the formation of complete and highly developed animals like bees, mice, frogs and the like from putrid matter, mud, cheese, dirty linen, etc.

(*c*) That which concerns the cause of putrefaction and fermentation.

Louis Joblot (1645–1723). After Leeuwenhoek's discovery it was thought by many who believed in the spontaneous generation of life that animal or vegetable matter contained a "vital or vegetative force," capable of converting such matter into new and different forms of life, and Leeuwenhoek's "animalcules" were hailed by many as proof of this. In 1710, Louis Joblot observed that hay, when infused in water and allowed to stand so for some days, gave rise to countless animalcules or infusoria (bacteria and protozoa). Many regarded this as conclusive evidence of spontaneous generation. Joblot, however, boiled hay infusion and divided it into two portions, placing one in a carefully baked and closed vessel, the other in an open vessel. The infusion in the open vessel teemed with living creatures in a few days. In the closed vessel no life appeared as long as it remained closed, thus showing that the infusion alone, once freed of life, was incapable of generating life anew spontaneously.

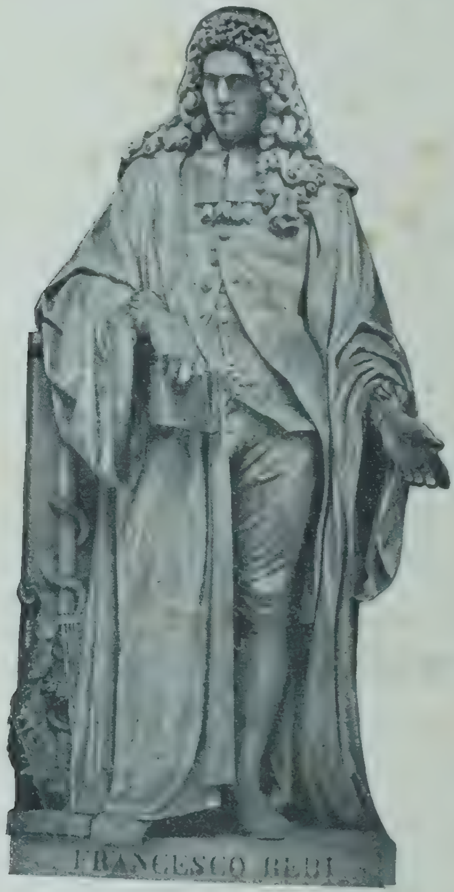

Fig. 5. Statue of Redi in the Portico degli Uffizi.

John Needham (1713–1781). Similar experiments, carried out by an English scientist, John Needham (1749), gave conflicting results. Life developed in Needham's heated, closed vessels as well as in the open, unheated ones. He, therefore, believed in spontaneous generation. We shall see later that this sort of result was probably due to insufficient heating, which failed to kill heat-resistant spores. But nothing was known about spores at that time.

Lazzaro Spallanzani (1729–1799). Spallanzani (Fig. 6), an Italian naturalist, published the results of a whole series of the same type of experiments, which disagreed with those of Needham. He showed that if dust and air are excluded with sufficient care, and if heating be prolonged sufficiently, and the vessels kept closed, no animalcules develop in hay infusions, or in any other kinds of organic matter like urine and beef broth. Needham, in reply, said the prolonged heating destroyed the "vegetative force" of the organic matter which, he said, was necessary for the spontaneous generation of life. Spallanzani answered Needham's objections by the very simple expedient of showing that the heated infusions in the closed flasks could still develop animalcules *when exposed to air.*

Fig. 6. Lazzaro Spallanzani.

In 1775 Lavoisier discovered *oxygen* and the relation between air and life and this renewed the controversy about spontaneous generation, the objection to Spallanzani's results being raised that it was the exclusion of air (oxygen) from the flasks which prevented the development of life.

Schulze and Schwann. New experiments were performed, in which air was admitted to the previously heated infusions of meat or hay, but only after passing through sulfuric acid or potassium hydroxide solutions (Schulze, 1836)[8] or through very hot glass tubes (Schwann, 1836),[8] their idea being that *the air itself introduced the germs of life into the infusions* (Figs. 7 and 8). When the infusions exposed to

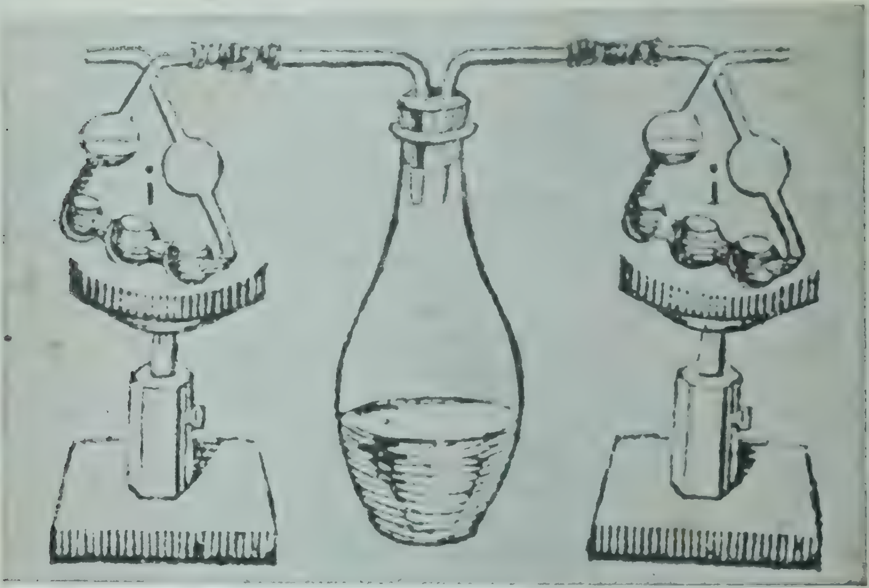

Fig. 7. Apparatus of Schulze for treating air before admitting it to flasks of putrescible material.

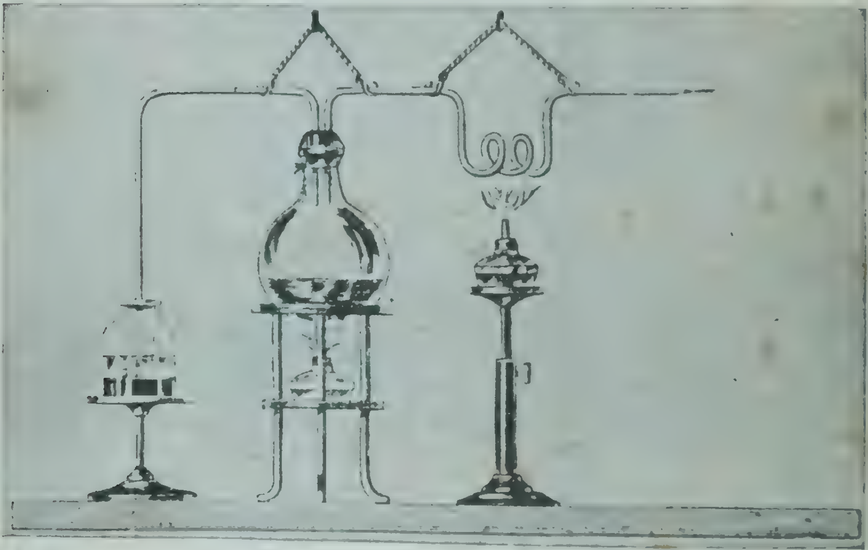

Fig. 8. Apparatus of Schwann for treating air before admitting it to flasks of putrescible material.

air so treated failed to develop any life, it was claimed by others that it was not due to a destruction of any germs of life in the air by the sulfuric acid or hot glass, but to the fact that the "life-giving" power of the air had been destroyed by these methods, thus preventing spontaneous generation. Schröder and von Dusch (1854–61)[9] overcame this objection by filtering the air only through cotton wool. This method prevented the appearance of animalcules in the heated broth or infusions until the vessels were opened and it then became apparent that the method of treatment of the air had nothing to do with the development of animalcules, *and that these did not develop spontaneously* but that there were particles of living matter in the air which not only were killed by heat, acids and alkalis but which could be caught and withheld by the cotton wool alone. The presence of the microorganisms in the cotton wool was later proven by Pasteur. The experiments of Schröder and von Dusch were the origin of our present-day use of cotton plugs for bacteriological culture tubes and flasks.

In spite of these demonstrations long and bitter controversies still raged. Schröder and von Dusch were themselves not convinced by their own experiments and admitted the possibility that spontaneous generation might occur under natural conditions.

Louis Pasteur (1822–1895). Pasteur, the most famous, and considered by many the greatest, French scientist of all time, was born in Dole, December 27, 1822. Son of a moderately prosperous tanner who had fought for, and been decorated on the battlefield by, Napoleon, Pasteur had a great admiration for his father's soldierly accomplishments. He later was moved to many of his best scientific achievements by his patriotic zeal. In his boyhood he was an indifferent student but later became an enthusiastic scholar, devoting his energies to a study of chemistry. He discovered the relationship of the stereo-isomeric forms of tartrate crystals and revealed a whole new series of possibilities in physical chemistry.[10]

Pasteur, however, was not one to gloat over such successes and rest on his laurels. He sought for other fields of investigation. His choice was guided largely by patriotic motives. An Englishman had said to him: "People are astonished in France that the sale of French wines should not have become more extended here [in England] since the Commercial Treaties. The reason is simple enough. At first we eagerly welcomed those wines, but we soon had the sad experience that there was too much loss occasioned by the diseases [souring] to which they are subject."[11, *] Germany was, at that time, making much better beer than France, and Pasteur undertook to make France a successful rival in that respect. In order to do so he made a long study of beer manufacture and of the cause of souring and spoilage ("diseases") of beer and wines. As a result of these studies he arrived at far-reaching conclusions. "Might not the diseases of wines," he said at the Académie des Sciences in January, 1864, "be caused by organized ferments; microscopic vegetations, of which the germs would develop when certain circumstances of temperature, of atmospheric variations, of *exposure to air,* would favour their evolution or their introduction into wines? . . . I have indeed reached this result, that the alterations ("diseases") of wines are coexistent with the presence and multiplication of microscopic vegetations" (Fig. 9). Pasteur had found that acid wines, "ropy" wines, bitter wines, sour beer and so on, were due to the growth in them of undesirable contaminating organisms which produced these so-called "diseases."[11, *]

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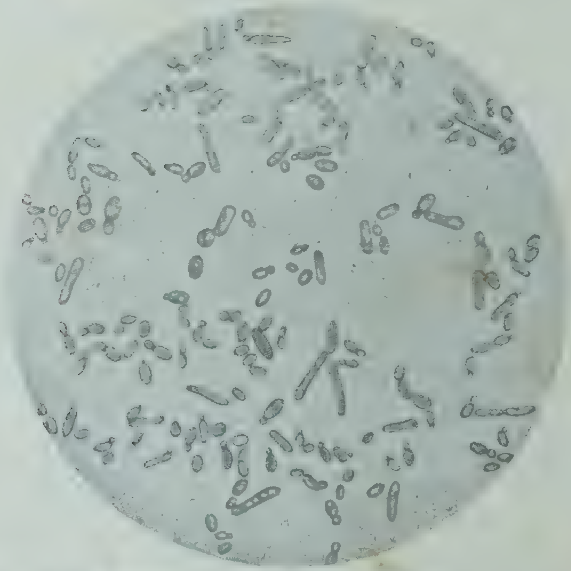

Fig. 9. Yeast, drawn by Pasteur himself. (Pasteur, Translated by Faulkner and Robb, Macmillan & Co., 1879.)

Pasteur soon became an authority on beer and wine manufacture and his advice and services were in great demand. "The town of Arbois, proud of its celebrated rosy and tawny wines, placed an impromptu laboratory at his disposal during the holidays of 1864; the expenses were all to be covered by the town."[11, *] Pasteur, however, fitted up his own laboratory.

The solution of the problem, according to Pasteur, lay in preventing the growth of foreign organisms, "wild" yeasts, bacteria, etc., which caused the undesirable conditions. After considerable experimentation along these lines (Fig. 10) he discovered that the diseases did not occur, and the wine did not spoil in transit, if it were held for some minutes at a temperature between 50° C. and 60° C. He said, "I have . . . ascertained that wine was never altered by that preliminary operation (heating), and as nothing prevents it afterwards from undergoing the gradual action of the oxygen in the air—the only cause, as I think, of its improvement with age—it is evident that this process (heating) offers every advantage." His experiments were so successful that a practical test of the efficacy of his methods was made. He wrote to a friend, ". . . experiments on the heating of wines will be made by the Minister of the Navy. Great quantities of heated and nonheated wine are to be sent to Gabon so as to test the process; at present our colonial crews have to drink mere vinegar."

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Fig. 10. Pasteur in his laboratory.

"A first trial was made at Brest on the contents of a barrel of five hundred liters, half of which was heated. Then the two wines were sealed in different barrels and placed in the ship *Jean Bart*, which remained away from the harbour for ten months. When the vessel returned, the Commission noted the limpidity and mellowness of the heated wine, adding in the official report that the wine had acquired the attractive colour peculiar to mature wines. The nonheated wine was equally limpid, but it had an astringent, almost acid flavour. It was still fit to drink, said the report, but it were better to consume it rapidly, as it would soon be entirely spoilt. Identical results were observed in some bottles of heated and nonheated wines at Rochefort and Orleans."[11, *]

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Pasteur laid down three great principles:

1. Every alteration, either of beer or of wine, depends on the development in it of microorganisms which are ferments of "diseases" of the beer or wine.
2. These germs or ferments are brought by the air, by the ingredients, or by the apparatus used in breweries.
3. Whenever beer or wine contains no living germs it remains unchanged.

In the same way that wines could be preserved from various causes of alteration by heating, bottled beer could escape the development of disease ferments by being brought to a temperature of 50° C. to 55° C. "The application of this process gave rise to the new word, 'pasteurized' beer, a neologism which soon became current in technical language." Today, pasteurization of milk (heating at 62° C. for thirty minutes) is commonplace. The heating kills the undesirable germs.

"Pasteur foresaw the distant consequences of these studies, and wrote in his book on beer—'When we see beer and wine subjected to deep alterations because they have given refuge to microorganisms invisibly introduced and now swarming within them, it is impossible not to be pursued by the thought that similar facts may, *must,* take place in animals and in man."[11]

It was obvious from Pasteur's studies that each special kind of fermentation or disease of beer or wine was the result of the growth and activity in it of a *special, distinct* form of yeast or other microorganism depending on the type of fermentation or disease under investigation. This furthered an idea, already old, of the *specificity* of biological action, and supported the view that animal and human diseases also, like different sorts of putrefaction and fermentation in wine vats, were each caused by a single, specific type of germ.

After Pasteur's views with regard to the nature of fermentation had been made public, he became involved in the bitter quarrel over the apparently mysterious appearance of the "germs" in fermentable or putrescible liquids like wine, beer, urine, broth, etc., hitherto regarded by many (Needham and others) as resulting from spontaneous generation. Without going into detail which would occupy too great a space, we may cite a series of experiments which Pasteur carried out to answer the various objections and fallacies of previous workers, and to show that the "animalcules" were merely descendants of germs which had gained access to the fluids from dust in the air and which, by their growth and metabolism, caused fermentation and putrefaction. First he redemonstrated that living creatures float in the air attached to particles of dust. Then he showed, as Schulze and Schwann had done, that when they could be excluded from various things like sterilized broth and urine, these substances did not ferment or putrefy. By using flasks with long, open necks having several vertical bends in them, he showed that, although unheated and untreated and unfiltered air communicated freely with the interior, the dust was caught by gravity in the bends of the neck and no life appeared in the infusions (Fig. 11). Not until the flask was tilted so that the fluid came into contact with this dust and was allowed to run back into the flask, or until the neck of the flask was broken off close to the body, did growth occur in the fluids. Some of Pasteur's flasks which were sterile in 1864 have been preserved and are still sterile (if they have not been destroyed by bullets) after 84 years! Nevertheless, Pouchet and Bastian still disputed loudly and long. The problem was taken up by the English physicist, John Tyndall.

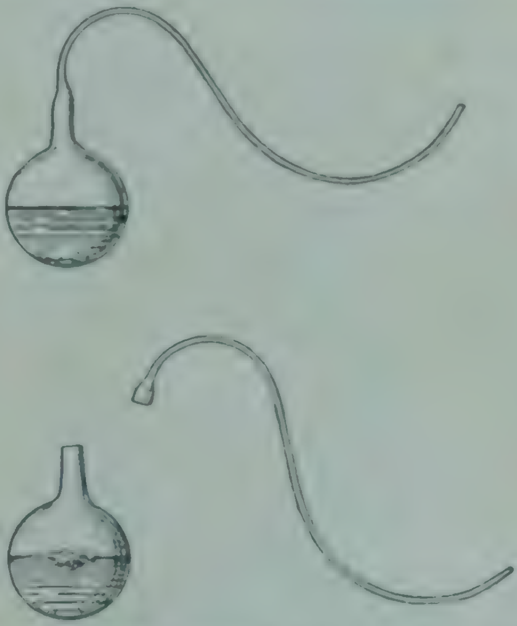

Fig. 11. One of Pasteur's flasks, showing open neck with curves to catch dust. (Duclaux, "Pasteur, The History of a Mind," W. B. Saunders Co., Philadelphia.)

John Tyndall (1820–1893). Tyndall and Pasteur were friends. To investigate spontaneous generation, Tyndall arranged a small cabinet with windows in two opposite sides and in the front, a door in the back, and several holes in the bottom through which test tubes were thrust. Openings were made in the top of the cabinet for the admission of air (through bent glass tubes like Pasteur's) and for the stem of a funnel. The interior of the cabinet was painted with glycerin which caught and held all dust particles as they settled so that after a time, when sunlight was passed through the cabinet from window to window, no "sun beams" (floating dust) could be seen (Fig. 12). The air was then said to be "optically pure." Tyndall now introduced mutton and vegetable broths into the test tubes through the funnel. The tubes were heated from below until they boiled for some time. They were left open and observed every day for many weeks. All remained sterile. They were perfectly capable of developing life, however, in spite of the heating, as shown by the fact that when dust was admitted to the cabinet all became putrid in a short time.

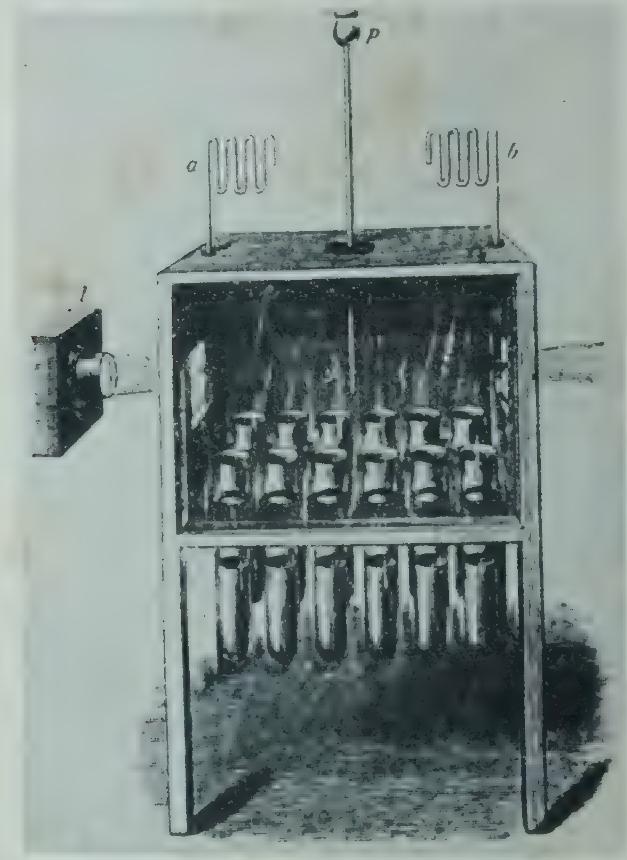

Fig. 12. Tyndall's cabinet for experiments on spontaneous generation.

Lord Lister (1827–1912). One of the most important applications of the work of Pasteur and Tyndall was made in 1867 by the English surgeon Lister (Fig. 13). He realized that wounds become infected, during surgical operations, by bacteria floating on particles of dust in the air, or clinging to instruments or to the skin of the operator (Fig. 14). Obviously, in order to prevent such wound infections it was only necessary to render all surgical appliances sterile and, by antiseptic dressings and careful technic, completely to prevent the entrance of bacteria into surgical wounds. Lister's original method of preventing infection during surgical operations was to work in a field and atmosphere continuously wet with a fine mist of antiseptic, carbolic acid solution, emanating from a nearby apparatus (Fig. 15). This must have made the surgeon's work difficult but the results obtained were

Fig. 13. Lord Lister.

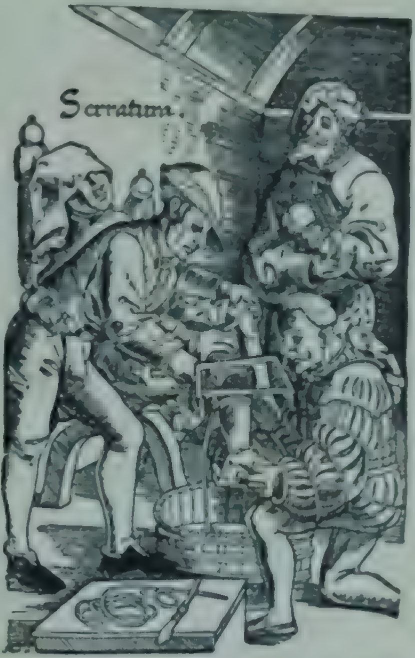

Fig. 14. First illustration of amputation, from Von Gersdorff (1517). Bandages constrict, at most, superficial veins. Two arteries are spurting uncontrolled. Man in background is wearing Gersdorff's pig's bladder dressing over his forearm stump.

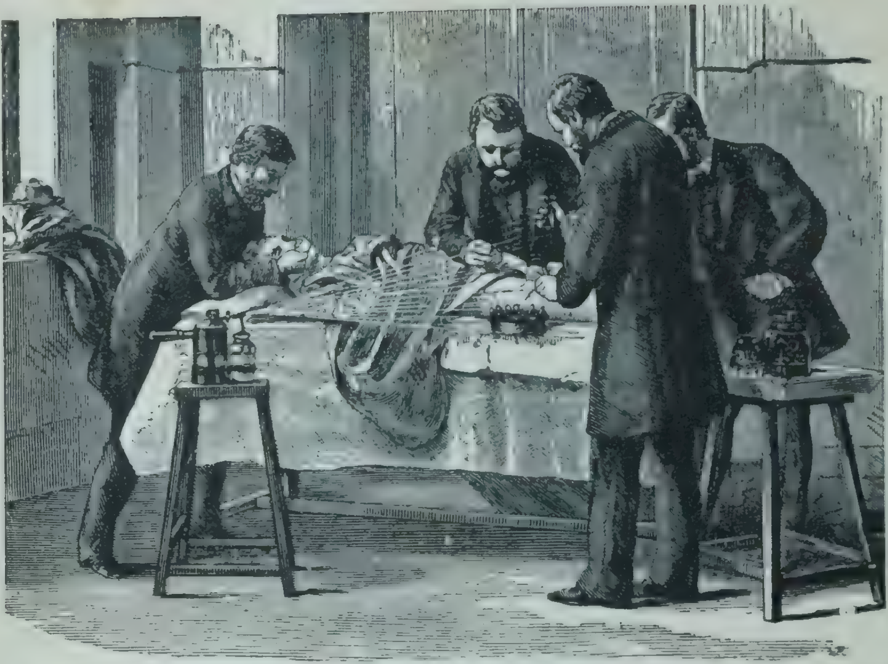

Fig. 15. Lister operating with carbolic spray. Representing the general arrangement of surgeon, assistants, towels, spray, etc., in an operation performed with (supposed) complete aseptic (antiseptic) precautions (1882). Note the carbolic spray playing over the field of operation. (W. Watson Cheyne.)

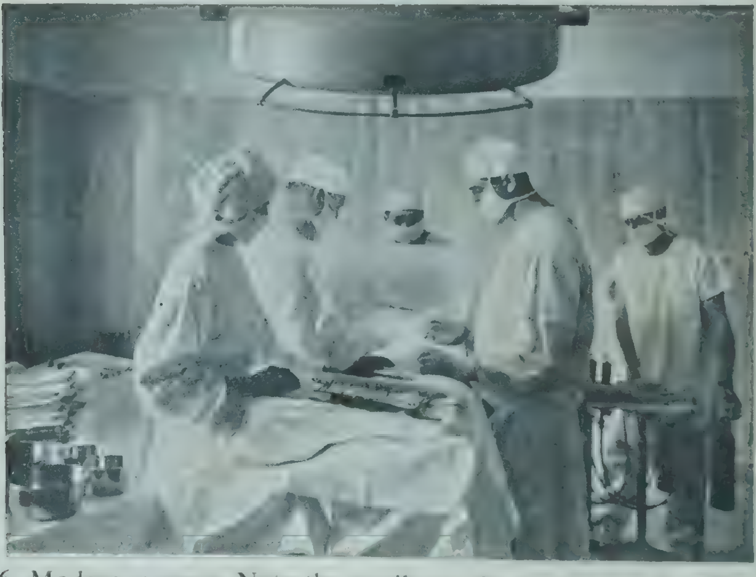

Fig. 16. Modern surgery. Note the sterile masks, caps, gowns, rubber gloves, sheets. The instruments have all been sterilized. This is a U. S. Government Hospital. (Baltimore Sun, courtesy of A. Aubrey Bodine.)

the foundation of our modern, aseptic surgery (Fig. 16). The student may judge for himself whether Lister's contribution was of importance to human life and medicine.

The Beginnings of Precise Bacteriology. One reason, perhaps, why more species of bacteria were not discovered by Pasteur and others prior to about 1870 was that culture methods were very crude. It was almost impossible, at that time, to separate, in a pure and uncontaminated state, any given sort of organism from a mixture of organisms. The preparation of such "pure cultures" is absolutely essential to the exact study of bacteria. For the development of pure culture technic, and for many of our modern bacteriological methods we are indebted to that precise German scientist, Robert Koch.[10, 12, 13]

Robert Koch (1843–1910). While Pasteur, Tyndall, Pouchet, Bastian and others were engaged in their polemics and experiments on fermentation and spontaneous generation, Koch was practicing medicine as a sort of "County Health Officer" in Wollstein. He had occasion, in an official capacity, to investigate anthrax (a disease of animals and man caused by certain, cylindrically shaped bacteria of the form called *bacilli*) and he decided to study the disease in his laboratory during his spare time.

At first Koch and other bacteriologists examined all their bacteria in the living state, usually in drops of fluid mounted on a bit of glass. They thus became familiar with bacterial motility when present, and they observed refractile granules inside various bacteria. But the constant motion of some of the bacteria (either that purposeless oscillation, due to molecular impact and known as brownian movement, or real, progressive motion due to the action of flagella) as well as their transparency, made accurate and prolonged study most difficult. Koch realized that it would be much better for his drawings, and especially for his photographs (both of which, by the way, were excellent; Fig. 17) if the bacteria could be made to remain still. He tried spreading out his drops of anthrax-infected fluid in thin films and allowing them to dry, and met with immediate success. Not only were the anthrax bacilli motionless, but they apparently had not shriveled or changed in any visible way. However, the bacteria were transparent and colorless. It was very difficult to observe the fine details of their structure, and equally difficult to photograph them. He obtained ideas from other workers, a procedure commended to all investigators.

Development of Staining Methods. Weigert and Ehrlich. Weigert, a German scientist contemporary with Koch, had observed the use, by Cohn and others, of various dyes to make clear the details of cell structures in histological preparations (histology = microscopic anatomy). This procedure had been in use for some time, the natural dyes *carmine* and *hematoxylin* being widely used. Ehrlich, a renowned chemist, had recently improved methods, discovered by William Perkin, of preparing very fine dyes from coal-tar distillates. These were the first "coal-tar" or aniline dyes. Weigert, the bacteriologist, tried the methods of the histologists with the dyes invented by the chemist, Ehrlich. His first success was in 1875, when he found that the dye *methyl violet* could be used to reveal bacteria in histological preparations. This method of making bacteria easy to find and study, where before they had been colorless and transparent and therefore almost invisible, was adopted by Koch and soon came into wide use. It is one of our best methods today.

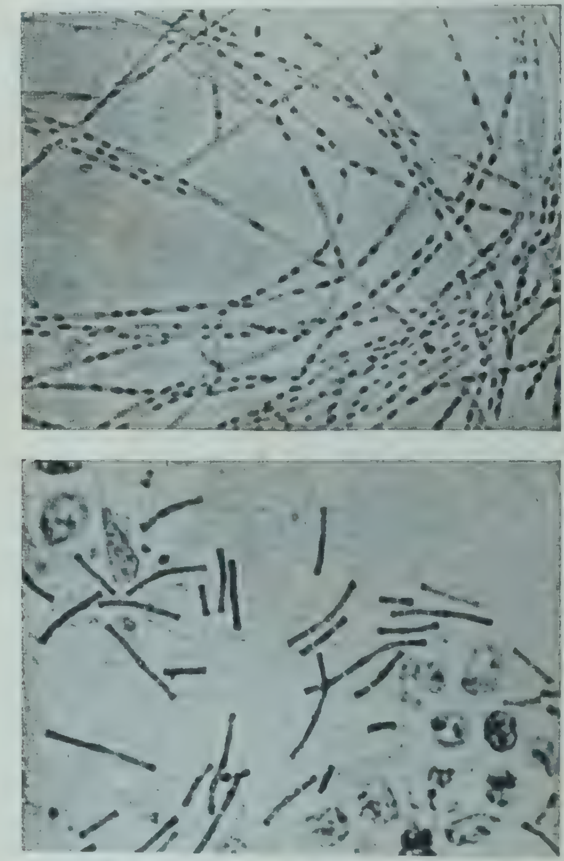

Fig. 17. Koch's photograph of anthrax bacilli.

Development of Gram's Stain. In 1884, a Danish scholar, Gram, found that if anthrax bacilli were stained with Ehrlich's methyl-violet solution, and then treated with iodine, the dye was "fast" and that even alcohol could not remove it. In attempting to apply the method to certain other bacteria, however, he found that the dye was sometimes not fast and that as soon as alcohol was applied the color was removed and the bacteria were as colorless and transparent as ever. They could even be dyed an entirely different color counterstained with some other dye. The bacteria retaining the purple stain were called gram-positive, while the others were called gram-negative. The method is used, generally with some slight modification, by every bacteriologist as one of the first steps in the process of identifying an unknown organism.

Koch, using the methods of Ehrlich, Weigert, Cohn,[14] Gram, Petri (see Chap. 7) and others, became one of the foremost bacteriologists of his day. His discoveries attracted scholars from all over the world (Fig. 18). In another chapter there is outlined the epoch-making development of solid media and the covered plate for the pure-culture cultivation of bacteria, which are methods used daily in every bacteriological laboratory in the world. The methods and principles which originated in the laboratories of Koch, Pasteur, Tyndall, Lister and others have made possible the conquest of disease and the building up of great industries. Greatest of all, they have left us a foundation of fact and inspiration upon which to build greater achievement and this, possibly, is the richest legacy which any man can leave to posterity.

Reading from left to right and from top row forward, the following persons are represented: Adamson (London), Burnett (Buffalo), Heine (Berlin), Mackenzie (Fall River, Mass.), Blesenick (Berlin), Ransom (Cambridge), Weber (New York), Crooker (Providence, R. I.), Dahmen (Koeln a R.), Straub (Brooklyn, N. Y.), Hargrove (Jacksonville, Ill.), Kestermacher (Stettin), Fricke (Münster i. W.), Scott (Edinburgh), von Szydlowski (Stanisiau), Sanderson (San Francisco), Bond Stowe (Chicago), Metternich (Mainz), Tobold (Berlin), Ruegenberg (Spandau), Weidl (Berlin), Hellebower (Cincinnati), Frank (Meiningen), Mannheim (Berlin), Sobernheim (Berlin), Scholl (Treptow a R.), Connell (Fall River, Mass.), Mlady (Meran S Tirol), Rosencrantz (San Francisco), Feigel (Lemberg), Pfeiffer (Berlin), Koch (Berlin), Frosch (Berlin), Stüler (Berlin), Costa Pruneda (Chile), Stagnitta (Messina).

Fig. 18. One of Koch's classes in bacteriology, 1881, in Berlin. Several of these men became world-famous. Koch is the bearded figure near center of front row.

Other Developments. Pasteur, Koch and their contemporaries laid foundation stones of basic importance in the science of Bacteriology and their names are writ large in the Halls of Fame.[15, 16, 17] However, it must not be supposed that they were the last great bacteriologists. They were among the *first*. Other workers, equally ingenious, learned and courageous, have carried on the work along an ever-increasing number of lines of investigation. Although the five years following Koch's demonstration of the value of solid media for isolating bacteria witnessed the discovery of the organisms causing diphtheria, cholera, erysipelas, lockjaw, pneumonia and a number of other diseases, discovery of new species was only a matter of patient plating out of suspected materials and was only one, rather limited, phase of the subject.

Possibly the greatest single discovery in human history was made when, in 1890, von Behring and Kitasato found that guinea pigs could be immunized by injections against tetanus and diphtheria toxins and that their blood then contained antitoxin which would protect other animals. The extension of this principle to other diseases was an obvious possibility and soon bore fruit.

In the realms of soil microbiology, great advances were going on simultaneously. In 1893 to 1894 Winogradsky demonstrated that atmospheric nitrogen is fixed in the soil by the anaerobic bacterium *Clostridium pasteurianum*. Aerobic organisms having a like property, *Azotobacter chroococcum* and *A. agile*, were discovered by Beijerinck in 1901. These organisms are of absolutely fundamental importance to the history of the human race since, without fixation of atmospheric nitrogen in the soil, there would be no human race at all. This rather deflationary fact will be explained later. Winogradsky, Beijerinck, Omeliansky and others during the decade following 1895 also worked out the processes by which the ammonia, formed in the soil by various biological processes, is oxidized by successive stages, first into nitrites by organisms of the genera *Nitrosomonas* and *Nitrosococcus*, and then into nitrates by bacteria of the genus *Nitrobacter*. In the latter form nitrogen is readily available to most plants of agricultural value and without it the human race might have had a very different history. The equally important transformations in the soil of sulfur, carbon and other elements were also studied by these workers.

In 1892 Iwanowski discovered the existence of a disease-producing agent (virus of tobacco-mosaic) which was invisible and not cultivable on inanimate media and which transversed filters capable of holding back the smallest bacteria. Many other viruses have since been discovered and great strides have been made in our knowledge of that class of organisms. For example, Walter Reed in 1900 to 1902 demonstrated not only the mode of transmission but the virus nature of the cause of yellow fever. Later, about 1933, workers of the International

Health Division of The Rockefeller Foundation developed a fully effective vaccine against yellow fever and to date it has been given to millions of persons. The very development of such huge beneficent organizations as The Rockefeller Foundation, The George Williams Hooper Foundation and the National Foundation for Infantile Paralysis, sponsored by the late President Roosevelt, to mention only a few, are in themselves tributes to the continued and growing value of work in the fields of bacteriology and microbiology.

One interesting and exceedingly valuable series of discoveries has resulted more or less directly from the studies of Koch, Ehrlich, Weigert and the others with the staining of bacteria. From continued investigation of the effects of dyes upon bacteria there has arisen knowledge of such dye-like compounds as sulfanilamide and sulfadiazine, the use of which in treating bacterial infections is a matter of common knowledge. In 1929 Fleming made the momentous discovery of penicillin, and Waksman et al., a little later, discovered streptomycin, thus opening up the huge field of antibiotics.

So Science grows. The history of tomorrow is being written in the laboratory notebooks of students today. And who knows which notes will prove to be the basis of the greatest discovery?

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Chapter 2

THE PLACE OF MICROORGANISMS IN THE ORGANIC SYSTEM

In order to orient the beginner in the world of microscopic creatures it may be well to discuss briefly some of the properties of these diverse types of microorganisms, such as protozoa, and to compare bacteria with these and with certain algae and related forms of life. It is assumed that the student already has a speaking acquaintance with both protozoa and algae and with the relations of these to more complex animals and plants.[1–7]

Bacteria and Protozoa. Not all of the minute living beings which a microscopist observes are bacteria. In water in which a bit of hay has been allowed to soak for a few days, for example, not only do many kinds of bacteria abound, but also various other fantastic and marvellously beautiful creatures which resemble bacteria in many respects but which can, as a rule, be readily differentiated by their relatively huge size, their elliptical or ovoid form and other distinctive features. These are protozoa (Fig. 19)—microscopic animals each consisting of only a single cell.

The protozoa are the most primitive members of the animal kingdom, just as most bacteria are of the vegetable kingdom. As contrasted with bacteria structurally the protozoa are very complex and usually contain well-defined portions performing the functions of organs in more highly organized animals.

The protozoa seem to be the results of development, by natural processes, of complexity of physiological function without increasing the number of cells. Thus, there is, in many free-living forms of protozoa, a portion of the cell which is somewhat thickened, rigid and even chitinous in composition. This serves as a skin or outer integument. This often contains or forms bristles (cilia or flagella) which have the functions of swimming appendages of higher animals. Although there are no proper muscles, there are certain portions of the protoplasm which seem to be contractile and may act the part of muscles in moving the cilia or flagella. The integument excretes waste products much as do the skin and kidneys of a human being, and thus serve the function not only of swimming oars, muscle and skeleton, but also of kidney, intestine and sweat glands. In so far the protozoa and bacteria have much in common. Inside the cell membrane of protozoa, however, there are usually a well-defined nucleus, nucleolus and cytoplasm, the last often containing various vacuoles which collect waste products eventually to be excreted through the cell wall, thus serving the purpose of bladder and colon of higher animals. Other vacuoles contain food particles in the process of digestion and thus are analogous to stomachs. Bacteria contain none of these. There are also granules of stored-up reserve material equivalent to fatty deposits or liver-glycogen reserves in mammals. Bacteria often store up granules of food reserve.

Although no such complex structures as nose, eyes, or nerves are found in protozoa, many protozoa are nevertheless quite sensitive to heat, chemicals, gravity, electricity, and light; and in one species at least (*Euglena*), a portion of the cell is differentiated into a specially sensitive area called a "light spot" which, although not a separate organ, serves the function of a primitive eye. There are some bacteria which possess these properties. Furthermore, protozoa in general accept or reject food particles or waste, and have quite a delicate sense of touch

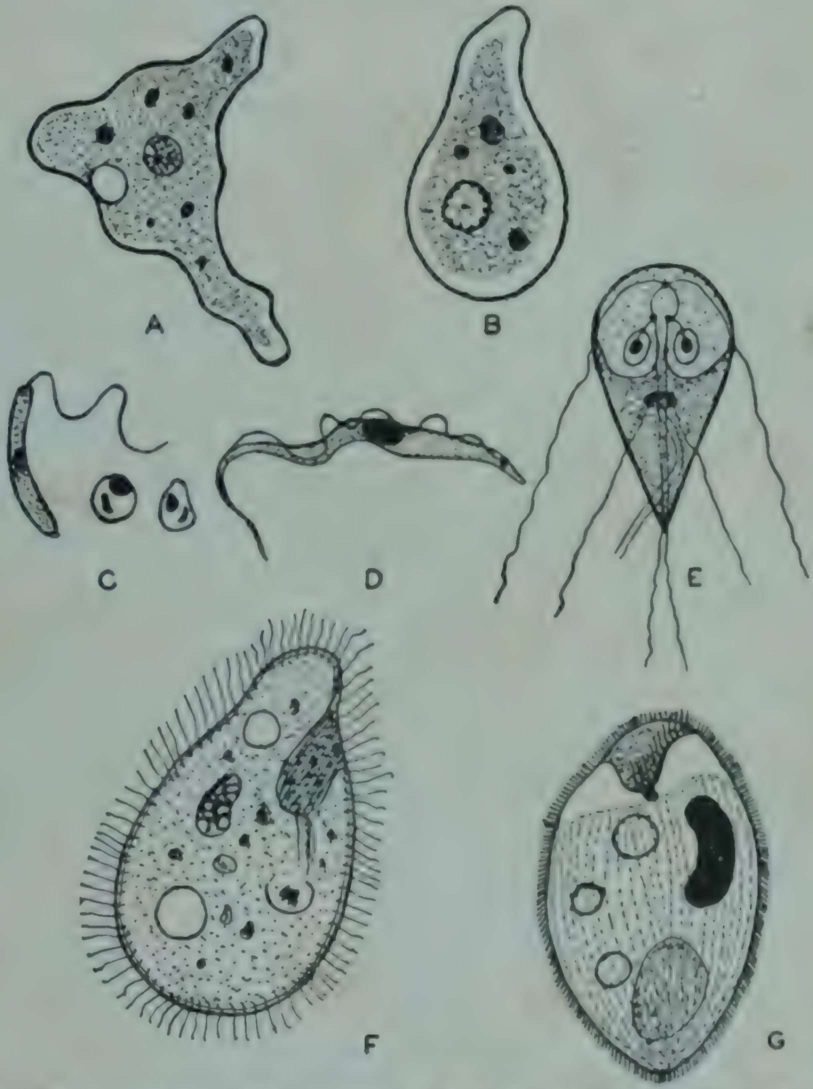

Fig. 19. Various forms of protozoa. *A, B.* Forms of amobea. *C, D.* Forms of trypanosome. *E. Giardia lamblia. F, G.* Types of ciliates. (After Hegner, Cort and Root, from Burdon. "Medical Microbiology," by permission of The Macmillan Co., publishers.) (See also Chapter 51.)

so that they recoil on contact with hard objects, turning aside quite as though they were highly sensate and responsive creatures; all of which shows the marvellous possibilities contained within a single microscopic droplet of protoplasm. Reproductive processes are similar to those observed in the higher animals, inasmuch as there are, in many genera, sexual phenomena, with conjugation, nuclear interminglings etc., in all essential respects analogous to the sexual phenomena related to fertilization in higher plants and animals. In protozoa, however, there are usually no special sexual organs. There are no well-defined sexual phenomena in bacteria.

One respect in which protozoa have an advantage over higher forms is that, for purposes of reproduction, an individual need only split itself in two. Among the flagellate protozoa this is usually done lengthwise, and each half receives half of each essential part of the original cell. Bacteria split transversely.

The cells of all living creatures possess this power of simple, sexless, or *asexual* multiplication. It is spoken of as *cell fission*. In the unicellular forms, each cell remains a separate organism whereas, in complex forms, different cells group themselves together as into "guilds" and tissues (the organs of the body, for example), each group performing one of the functions found in single-celled animals, but all the groups cooperating to form and maintain a complex society of cells, or organism, consisting of these large masses of cells having highly differentiated functions. In the single-celled organism cell fission leads to the formation of new individuals; in many-celled organisms cell fission generally adds only to the size of the individual of which the cell is a part.

If we examine the animal kingdom as a whole, we find man at the top of the list, representing the latest and most complex development in organic evolution. Proceeding downward we pass through groups of lower animals less and less highly developed functionally and structurally and finally come to the protozoa, the least complex of all and the most primitive in an evolutionary sense. Only among the protozoa do we find any forms in the least resembling any of the bacteria, and this resemblance is not morphologically confusing and is rather remote. It will be discussed farther on.

Bacteria and the Vegetable Kingdom. Let us now examine the vegetable kingdom as a whole and consider the different divisions and their relations to each other and to the animal kingdom, especially the protozoa. Opinions differ as to the most logical method of classifying the organisms of the vegetable kingdom and no system can be said to be the best for all purposes. For the present, the schema given below is a convenient arrangement.

THE VEGETABLE KINGDOM

Phylum I. Tracheophyta (vascular plants). Contain chlorophyll.
  Subphylum 1. Pteropsida. Ferns and seed plants.
    Class 1. Angiospermae or Spermatophyta (flowering plants).
      Subclass 1. Monocotyledoneae (plants with 1 seed leaf)—lilies, grasses, etc.

Subclass 2. Dicotyledoneae (plants with 2 seed leaves)—roses, zinnias, maples, etc.
  Class 2. Gymnospermae (naked seeds)—pines, etc.
  Class 3. Filicineae—ferns.
 Subphylum 2. Sphenopsida—horsetails.
 Subphylum 3. Lycopsida—club mosses.
Phylum II. Bryophyta—Liverworts and true mosses.
  Class 1. Musci—mosses—*Sphagnum, Polytrichum*, etc.
  Class 2. Liverworts—*Marchantia*, etc.

Fig. 20. A very large alga. A large kelp (*Pelagophycus*) from the California coast. (Photograph furnished by W. A. Setchell. Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," John Wiley & Sons, Inc.)

Phylum III. Thallophyta—no roots, stems, leaves or flowers.
 Subphylum 1. Algae—contain chlorophyll.
  Class 1. Chlorophyceae—Green algae.
  Class 2. Rhodophyceae—Red algae.
  Class 3. Phaeophyceae—Brown algae (and Diatoms?).
  Class 4. Myxophyceae—Blue-green algae.
 Subphylum 2. Fungi—Do not contain chlorophyll.
  Class 1. Basidiomycetes—mushrooms, puffballs, tree fungi, etc.
  Class 2. Ascomycetes—yeasts, blue molds, mildews, etc.
  Class 3. Phycomycetes—"bread molds," etc.
  Class 4. Fungi imperfecti—heterogeneous asexual fungi of classes 2 and 3.
  Class 5. Schizomycetes—fission fungi, bacteria.

Phyla I and II may be disposed of as including only macroscopic green plants of relatively complex structure and showing well-developed sexual differentiation. None of these morphologically resembles bacteria or protozoa.

Phylum III, subphylum 1, contains many plants of relatively simple structure. Flowers and seeds are unknown. In spite of the fact that some of them (as the kelp-algae) may attain very large size (Fig. 20) (Macrocystis—600 feet in length) none is possessed of any well-differentiated root, stem or leaf structure, although certain seaweeds approach this complexity. Sexual phenomena are reduced to very simple forms.

Also occurring in each class are very minute and extremely simple plants in which even the primitive communal tendency described above is lost and each cell lives entirely disconnected from all others; each cell division resulting in a new individual, or at most in a loose pair, trio, or tetrad, whose members are easily separated by mechanical agitation. Among the algae *Synechococcus, Gloeocapsa,* and *Scenedesmus* are examples of this form of plant (Fig. 21). However, all of these resemble higher plants in cell structure and in their green color.

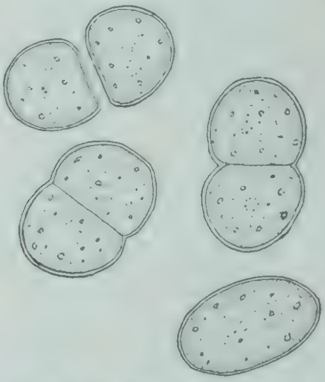

Fig. 21. *Synechococcus*, a blue-green alga the cells of which are solitary except during fission. Several stages of fission are shown as well as a single cell about ready for division. (Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," published by John Wiley & Sons, Inc.)

Some varieties of the green algae have cells (*Ulothrix* gametes, Fig. 22) which resemble protozoa in form, size and motility, but differ in possessing the green, sun-light-using coloring matter *chlorophyll*, which is common to these and all plants above the fungi.

Turning our attention to the fungi, we are struck by the absence of chlorophyll from all. In the group of fungi are organisms which do not resemble ordinary plants in size or appearance and which have no green color and are indifferent to, or even killed by, sunlight. The simplest of all of these are the bacteria.

Like other fungi, which probably evolved from bacteria, they lack the power of utilizing sunlight, and make use of chemical energy to

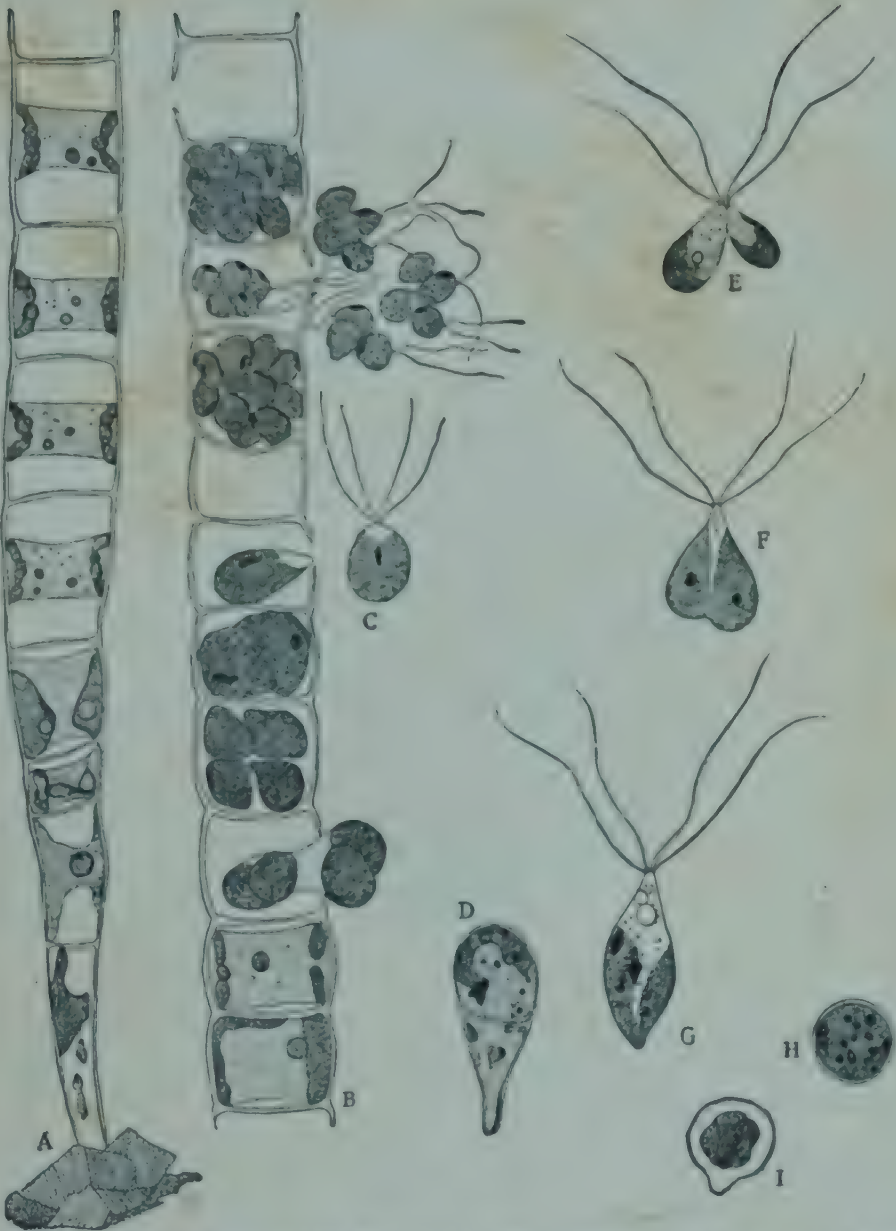

Fig. 22. *Ulothrix*, a green alga showing motile, protozoan-like gametes. *A*. Portion of vegetative filament. *B*. Formation of gametes (above). *C*. Zoospore with four flagella. *D*. Germination of zoospore. *E*, *F*, *G*. Conjugation of gametes. *H*. Resting zygospore. *I*. Growth and germination of zygospore. (Reprinted by permission from Holman and Robbins, "Textbook of General Botany," John Wiley & Sons, Inc.)

synthesize their cell substances from simpler compounds. A wide variety of chemical devices for this purpose is found in different species of bacteria, but they have in common the fact that synthesis can proceed independently of solar energy, the energy being derived from various chemical reactions rather than from sunlight. The process is therefore called *chemosynthesis*. In this respect bacteria resemble animal cells.

Among the larger fungi, such as mushrooms, bracket fungi and puffballs, or the wooly molds seen on decaying organic matter in warm weather, there are rudiments of sexual differentiation.

When we descend to the bacteria we encounter fungi only 1 or 2 microns in diameter; they exist as single-celled individuals or at most only as small, easily-broken-up agglomerations of independent cells; devoid of sex, without definite nucleus; multiplying into individual plants by simple fission; and hence called the fission fungi or Schizomycetes (Fig. 23).

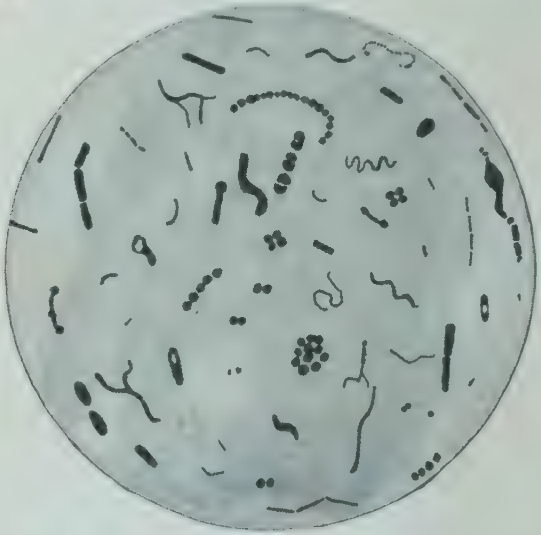

Fig. 23. Various forms of bacteria (× 900).

Are Bacteria Plants? Although all bacteria are included in the vegetable kingdom, it is extremely difficult to justify this method of disposing of them *as a group* because so many of them possess characteristics which are found in microscopic animals. Thus, some bacteria (*e.g. Acetobacter xylinum*) are plant-like because their cell walls consist of cellulose, a characteristically vegetable substance, but this is not true of all, or even of most. Some bacteria are said to possess cell walls of chitin, a distinctly animal substance which is the basic material of horn, hair, hoof and insect shell; but again, this is not generally true. The fact is that, because of the small size of the bacteria and the difficulties in their chemical study, exact information on this point is lacking.

Plants are sometimes said to differ from animals generally because the former are not motile while the latter are actively motile. With respect to large plants and animals and even among many of the very small forms this is generally true. Among bacteria, however, there are many motile as well as many nonmotile forms.

The simplicity of structure of the bacterial cell has been advanced as a basis for their classification as plants. This "simplicity," however, is merely a result of our inability to see the internal structures of the bacterial cell with ordinary microscopes. Photographs made with electron microscopes and phase microscopes (see Chapter 3) reveal structural details of very great complexity indeed. Further, simplicity is not an outstanding character of plant cells.

The presence of starch in a living cell is, in itself, strong evidence of the vegetable nature of that cell, because the formation of starch is almost exclusively limited to the vegetable kingdom. On the other hand, glycogen, a starchlike compound, is almost exclusively limited to the animal kingdom, and cells containing it may be regarded as of an essentially animal nature. However, a polysaccharide which seems to be indistinguishable from glycogen is said to have been extracted from sweet corn. There are several common species of bacteria which build up granules of starch within their cells, and others which synthesize glycogen. Still others synthesize peculiar substances resembling starch, but neither true starch nor glycogen, and called "granulose" or "iogen." The composition of the reserve carbohydrate substance of bacteria is, therefore, only a source of confusion when advanced as evidence for the vegetable nature of all the Schizomycetes.

In some respects the nuclear structure of bacteria is thought to resemble the diffuse nucleus of the Myxophyceae or blue-green algae. This may be so but there is still controversy as to the exact nature of the bacterial nucleus, due to the limitations of available optical methods. The bacterial nucleus certainly seems to be quite distinct from that found in cells of the animal kingdom and all other plants except the blue-green algae.

Manner of Taking Nourishment. All members of the vegetable kingdom (with a few exceptions) differ from all animals (with a few exceptions) in the manner in which food substances are taken into the body. Animals are able to take *solid masses* of food *into the body* (be it a many-celled or a single-celled body), there digesting it and turning it into soluble substances which nourish the cell or cells of the animal.* It is obvious enough in large animals, but may readily be observed in the smallest forms such as ameba or paramecium with the aid of a microscope. With a few exceptions, like "Venus's fly trap," the food of all plants, from the apple tree to the fern, sea-weed or bacterium, is absorbed through the cell walls of root or thallus, and this absorption can occur only when the food is in the form of relatively *simple compounds soluble in water.* The plant cell cannot ingest solid food.

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* If we consider the *tissue cells* of large animals, even this difference largely disappears.

This restriction in the manner of taking in food is one universal property of bacteria which links them most strongly to the vegetable kingdom; yet even here we find that it is not an exclusively vegetable trait since some parasitic protozoa (trypanosomes, malaria parasites) take in food much as do plants. However, with these few exceptions, nutrition only by diffusing soluble substances through the cell wall is probably the most generally vegetable-like property of the Schizomycetes.

The manner of taking food imposes some restrictions in the dietary of plants. Virtually all green plants can live on a wholly inorganic diet; a weak solution containing sodium nitrate ($Na\,NO_3$), potassium phosphate (K_2HPO_4), ammonium sulfate [$(NH_4)_2SO_4$], calcium chloride ($CaC1_2$) and a half dozen or so other mineral salts combined with a little oxygen and carbon dioxide from the air serving very well for breakfast, dinner and supper; a life-long, never ceasing diet of salts! Yet from such materials we derive potatoes, watermelons, wood, paper, and thousands of drugs, paints, oils, perfumes and the like. A metabolic system adjusted to such a diet is said to be *autotrophic* or *prototrophic* and is a characteristically vegetable arrangement. Animals, in contrast, require complex organic substances derived from the bodies or wastes of plants or other animals, and this kind of metabolic adjustment is spoken of as *heterotrophic.*

Autotrophic metabolism is a most distinctive vegetable trait, while heterotrophism (utilization of organic carbon sources) is a definitely animal characteristic; yet, while many bacteria are autotrophic, some of these same autotrophic forms can also live a distinctly heterotrophic existence, and many forms, especially disease-producing species, are obligate heterotrophs.

The exact systematic position of bacteria in the animate world, therefore, seems to be somewhat doubtful at present. Possibly some species should belong to the animal kingdom, some are undoubtedly vegetable in nature, while others may belong in an entirely new kingdom, neither animal nor vegetable—a sort of buffer state between the major taxonomic domains. However, it is not necessary that we settle the question before proceeding further with our studies.

Other Microorganisms. Three other groups of organisms must be described briefly in this place for purposes of orientation and comparison. They are classified in neither animal nor vegetable kingdom. These are the viruses, rickettsiae, and pleuropneumonia-like organisms. The first two are obligate parasites capable of multiplying only inside of other living cells and are, therefore, notable for their relationship to disease. Their pathologic aspects and other special considerations concerning them will be given in later chapters. The pleuropneumonia-like organisms have properties of both bacteria and viruses.

Rickettsiae. These organisms morphologically resemble true bacteria and are like cocci (spheres) or bacilli (rods) in form. They are much smaller than bacteria, however, and some of the smallest are almost at the limit of visibility with ordinary microscopes. Their structural details are very little known although the electron microscope seems to reveal at least a cell membrane. Of their biochemistry we know little or nothing. They appear to contain the same elements as other living matter, and to be made up of amino acids and other compounds common in protoplasm. They do not, so far as is known, live free in nature and they cannot, like bacteria, yeasts and molds, as will be described later, be made to grow in such lifeless materials as urine, milk, meat soup, etc. Hence little or nothing is known of their metabolic processes.

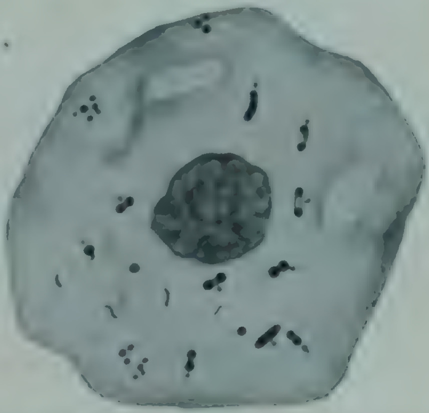

Fig. 24. Epithelial cell showing rickettsiae in the cytoplasm. Typical rickettsiae are indicated by arrows. (About 3000 ×.)

Whether they are plants or animals cannot be stated. It may be that, by extension of the evolutionary process of parasitism, they are derived from bacteria which have lost all ability to live outside the host cell.

Viruses. These are even more mysterious than the rickettsiae. They do not live free in any known natural environment and are associated only with disease. Unlike rickettsiae they are not visible at all by means of ordinary microscopes although they can probably be made visible by means of the electron microscope. Some are spherical or oval; some are crystals. Their internal structure is unknown. Like rickettsiae they are obligate intracellular parasites and cannot be cultivated in lifeless media like bacteria, yeasts or molds. Nothing is known of their metabolism. Some may not even be living beings. They pass through filters of porcelain so fine as to prevent the passage of rickettsiae and bacteria. They are not affected by sulfa drugs, penicillin or streptomycin as are many bacteria.

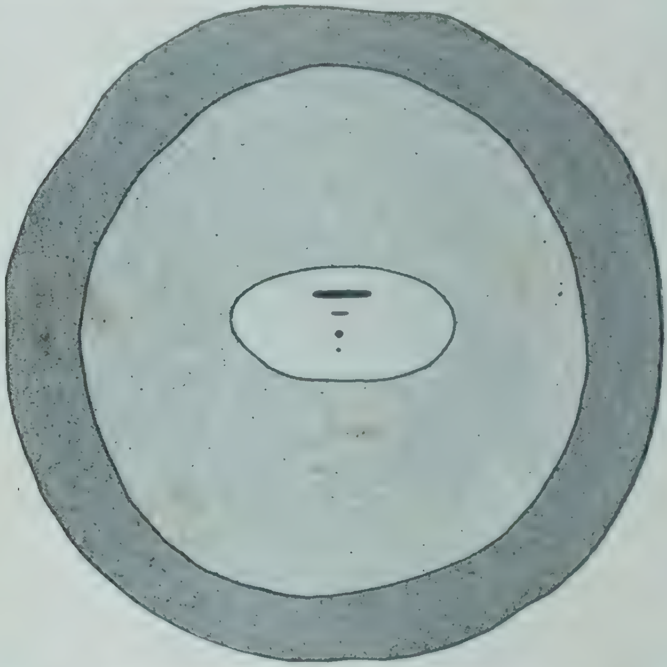

Fig. 25. Diagram showing relative sizes of microorganisms. In the center, the smallest speck represents the smaller viruses, such as yellow fever virus. The next larger objects represent, respectively, pleuropneumonia organisms, rickettsiae, a bacterium such as Escherichia coli. The ovoid white area next in size represents a form of yeast cell. The light grey area beyond represents a large protozoan, and the dark area outside an algal cell. There are, of course, many variants in size in all groups. This is merely a suggestive diagram.

Their place in the organic system is unknown. Like the rickettsiae, they may be derived from bacteria by extension of parasitic habits, or they may (most unlikely) be degraded protozoan forms. It is not inconceivable that they represent parts of injured host cells given off and in some way retaining the power of multiplication if given an opportunity in another cell. There are reasons for relating them to enzymes and genes. They are discussed more in detail later on in this book.

Pleuropneumonia-like Organisms. These are said to be stages in the life cycle of bacteria, but this is doubted by many. They exhibit cyclical changes in form from virus-like bodies to bacteria-like cells. The virus-like bodies are filterable. Unlike viruses, these organisms are cultivable on lifeless media. They are often associated with disease, but saprophytic species have been found in soil, sewage, etc.

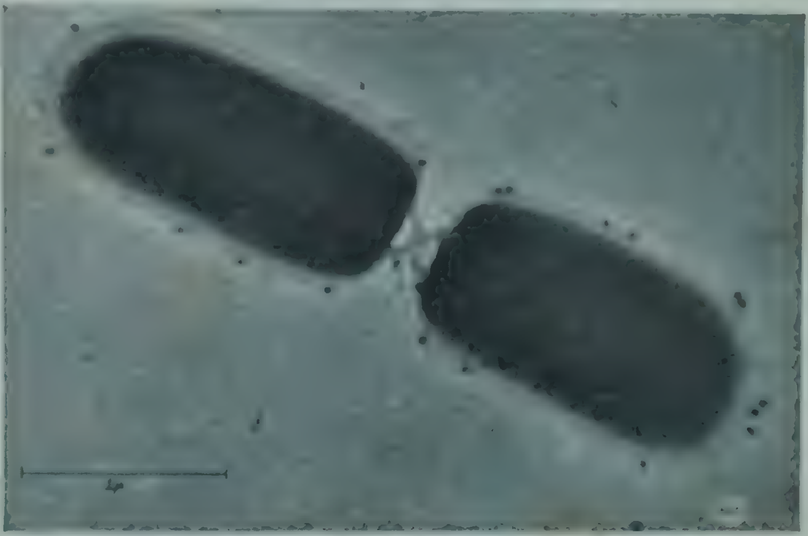

Fig. 26. Electronograph showing virus (bacteriophage) particles attacking two small bacteria (Escherichia coli). (S. E. Luria, M. Delbruck and T. F. Anderson, J. Bact., 1943.)

The relations of the protozoa, algae, bacteria, yeasts, molds, viruses, rickettsiae and pleuropneumonia-like organisms are shown in the table on page 183.

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Chapter 3

OPTICAL METHODS IN MICROBIOLOGY

Very early microscopists, about 1680, used simple, single lenses. These had very definite limitations due to their crude construction. In attempts to see still more, better microscopes have been built, until today we have very complicated, compound instruments which give magnifications of from two to ten times those of the best of the early instruments (up to 3000 diameters).

Complete description of the optical system of compound microscopes is not regarded as necessary here. Any good, modern college textbook of physics gives these details. However, a diagram showing the principal parts, as well as the path of the light rays through the instrument (Fig. 27), is included for the convenience of the student. A few directions for the use and care of the oil immersion lens may, in addition, not be out of place, as the bacteriologist uses this most of the time.[1, 2, 3]

An oil immersion objective lens magnifying about 90 to 100 diameters is generally used. The ocular or eyepiece should magnify 10 diameters. A total magnification of between 900 and 1000 diameters thus obtained is most useful, higher magnifications tending to give "fuzzy" outlines.

Use of the Microscope. It is usually a little difficult for a beginner to obtain good results with an oil immersion lens. A reliable procedure is as follows: a bright source of light is needed, such as a 75 watt "frosted" electric lamp, a bright, unclouded sky (not direct sunlight) or some special form of lamp of which there are several types on the market.

Have the *stage* level. With the low power or the "high dry" lens centered about ⅛ inch above the condenser, apply the eye to the *ocular* and then adjust the *mirror* and *diaphragm* so that a maximum of light is thrown up through the *barrel* of the instrument. Raise the barrel of the microscope with the large wheel and swing the *oil immersion lens* into center, being sure it snaps into position. Put a drop of *cedar* oil on the smear to be examined. Mineral oil is to be avoided because the image will be somewhat distorted. Certain commercial substitutes are far superior. After applying the oil place the slide, *smear upward*, upon the stage, with the smear over the center of the *condenser* lens. Now, using the large wheel (coarse adjustment) lower the barrel,

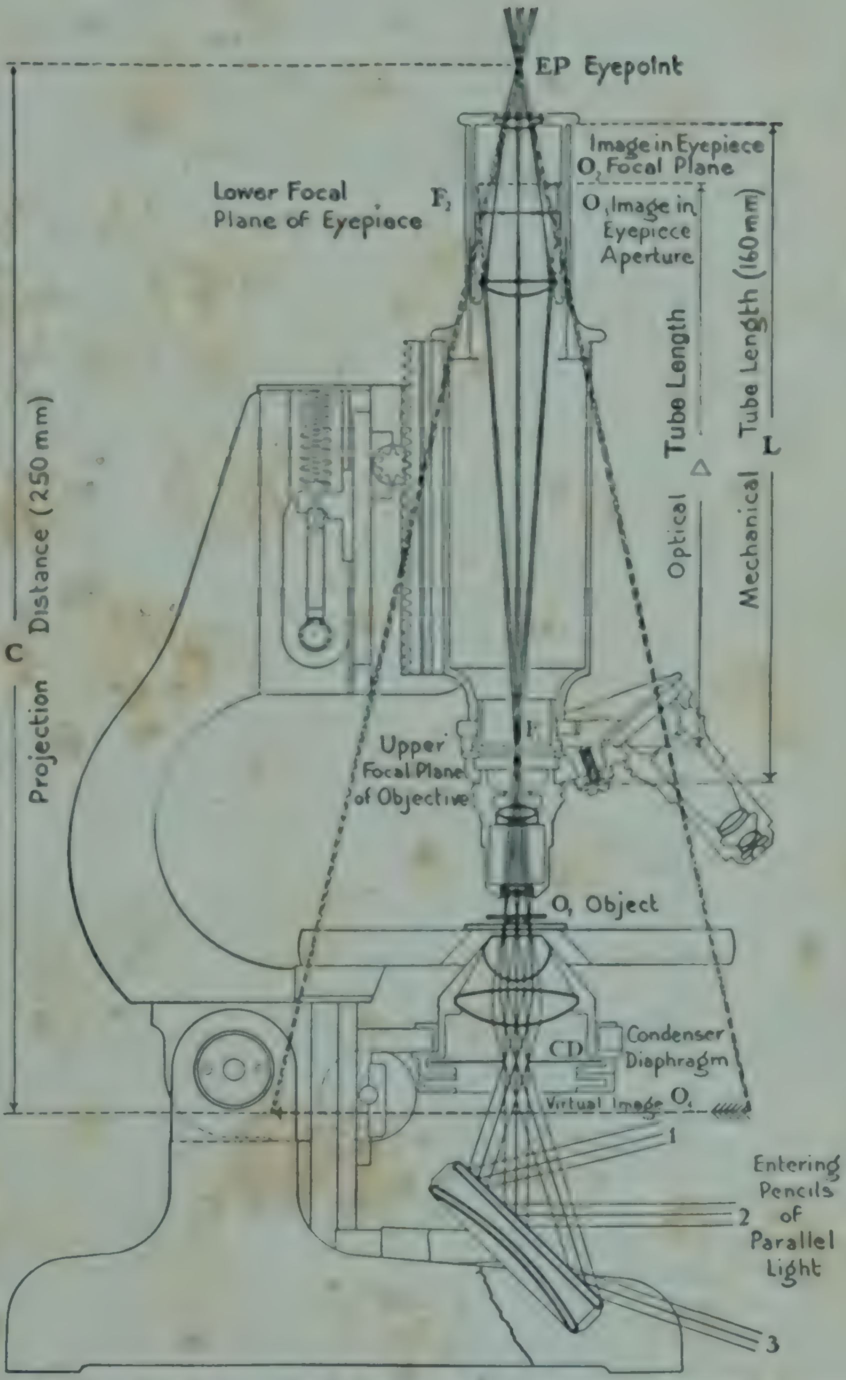
EP Eyepoint
Image in Eyepiece
O_2 Focal Plane
Lower Focal Plane of Eyepiece
F_2
O_1 Image in Eyepiece Aperture
Optical Tube Length
Δ
Mechanical Tube Length (160 mm)
L
Projection Distance (250 mm)
C
Upper Focal Plane of Objective
F_1
O, Object
Condenser Diaphragm
CD
Virtual Image O_4
1
2
3
Entering Pencils of Parallel Light

Fig. 27. Chart showing path of light through laboratory microscope. (Courtesy Bausch & Lomb Optical Co., Rochester, N. Y.)

watching (*from the side*) the lowest point of the objective until it touches and is immersed in the oil and *almost* touches the smear itself. *It should not touch the smear.* With the eye at the ocular, very slowly raise the barrel with the large wheel until the smear comes into view. The small wheel may be used when the smear is very nearly in focus, or when it temporarily gets out of focus during manipulation. The mirror and diaphragm may now be adjusted more accurately. Sometimes it is desirable to close the latter a little to obviate glare and give more detail.

Never lower the barrel with the large wheel unless you are watching the objective tip closely; otherwise, you will mash the slide and may ruin the lens. If you have difficulty, it may be due to the following causes: (*a*) slide upside down; (*b*) light insufficient; (*c*) bad adjustment of mirror or diaphragm or both; (*d*) use of the wrong objective or objective not centered; (*e*) dirty lenses (oil hardened on the objective should be removed with a piece of lens paper of soft cotton moistened with xylol; (*f*) poor focusing (try refocusing for practice); (*g*) condenser and substage not fastened up tight and close to the slide; (*h*) smear not under objective lens.

If you still have difficulty after checking up on these points, consult someone experienced in handling microscopes. Never attempt to repair your microscope or remove any lenses yourself. Keep your instrument clean and covered to keep out dust. Always remove immersion oil from the lenses immediately after use. Soft cotton, linen or lens paper should be used for this.

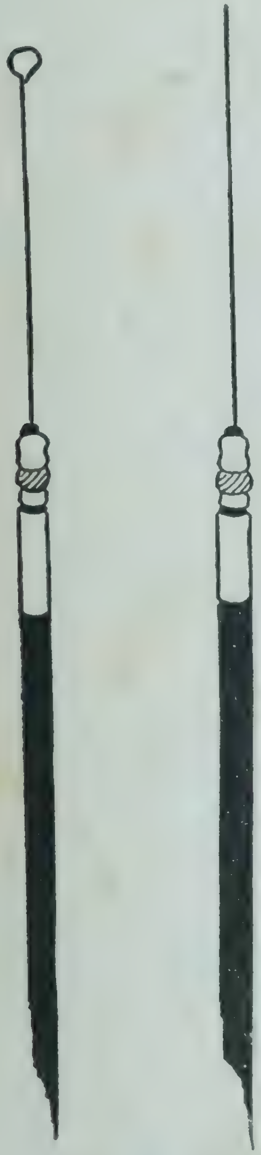

Fig. 28. Wire loop and needle used for transferring bacterial cultures.

"Hanging Drop" Preparations. Having become proficient in the use of the oil immersion lens, we may turn our attention to the things to be examined with it. If it is desired to examine bacteria in a natural, living state, they are best viewed when suspended in a clear fluid of some sort; usually water, saline solution or broth. The cells are transparent, colorless and refractile and so tiny that they are often difficult to find and even to identify as bacteria in the drop of fluid. This is especially true of the spherical types (cocci).

To observe bacteria in this state it is necessary to put a loopful* (Fig. 28) of the fluid in which they are suspended, on a thin cover slip. On each of the four corners place a droplet of mineral oil. Hold a "hollow-ground" slide, depression down, over the drop and bring the two into contact. Invert the slide quickly so that the drop cannot run off to one side. A *tiny* additional drop of clear mineral oil may now be run under the edges of the cover slip at each corner if needed. This spreads under the cover slip and prevents drying of the drop (Fig. 29).

In attempting to focus upon the drop, the diaphragm should first be closed about two thirds. Bring the low power lens into center. The low power lens is useful to locate the edge of the drop, which is usually easiest to find and bring into focus. The high dry lens may then be brought to bear upon the drop, and then, if necessary, the oil immersion. The latter is seldom necessary for inspection of the hanging drop.

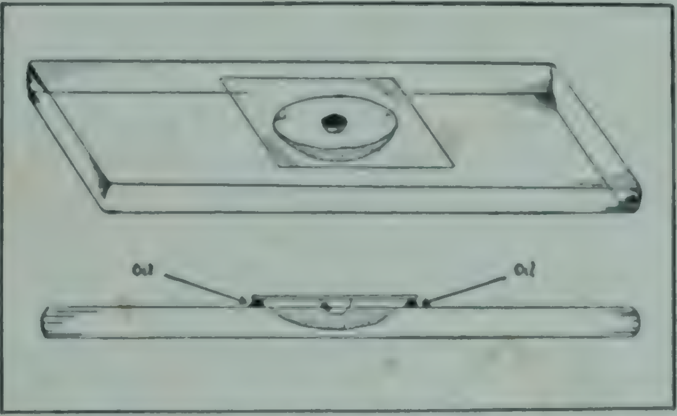

Fig. 29. Hanging drop preparation. The size of the oil droplets is greatly exaggerated.

In the hanging drop we may see the size, shape and arrangement of bacteria and their motion if they are motile. Sometimes bright, refractile granules and spores may be seen within the living cells. Due to the fact that bacterial cells contain no chlorophyll and are colorless and transparent it is extremely difficult to study them since this lack of color prevents details from showing clearly. Further, when suspended in fluid, they move about, either because of their own motility, currents in the fluid, or brownian movement. The observation of bacteria in hanging drop preparations, therefore, yields only a limited amount of information.

Preparations of Smears for Staining. To overcome this difficulty,

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* By a "loop" is meant the space included by a tiny ring or loop made at the end of a thin wire. The wire is fixed into some sort of handle so that it may be sterilized in a flame. Loops are usually about 2 mm. in diameter and a loopful is a small drop.

Koch's device of immobilizing bacterial cells and coloring them with aniline dyes is generally used. In order to accomplish this, the material containing the bacteria (soil, urine, pus, milk, feces, saliva) is diluted with water, saline solution or broth. A small drop of the suspension is spread evenly in a thin film, with a loop, over an area about 1 cm. in diameter. Once the loop has touched the slide, make the smear with a horizontal rotary motion, and then lift the loop *slowly* from the slide only when the smear is finished. *Flame the loop* before putting it down. Flame it also just before use, but *be sure it is cool* (twenty to thirty seconds in air) before applying it to the bacteria.

Having made the smear, allow it to dry, and then heat slightly for two to three seconds over the Bunsen flame. This makes the bacteria adhere to the glass. The procedure is called *fixing. Do not overheat!*

Nature of Dyes. Dyes used in microbiology may be thought of as salts of two kinds: (1) those in which the color-bearing ion (called the chromophore) is the anion, e.g., sodium$^+$ eosinate$^-$ (eosin); (2) those in which the chromophore is the cation, e.g., methylene blue$^+$ chloride$^-$. The first are acidic dyes in the sense that, as an acid, the chromophore combines with a base (NaOH) to form the dye salt; the second are basic because the chromophore acts like a base, combining with an acid (HCl) to form the dye salt.

In general, acidic dyes combine more strongly with cytoplasmic (basic) elements of cells; the basic dyes with nucleic (acidic) elements of cells. Bacteria are best stained with basic dyes.

For convenience in preparing dyes for staining, the following list of solubilities of commonly used dyes is given. (Adapted from Conn.)[4]

	Percent soluble (at 26° C.)	
	Water	*95% alcohol*
Bismarck brown	1.36	1.08
Congo red	0.	0.19
Thionine	0.25	0.25
Methylene blue (Cl)	3.55	1.48
Neutral red (Cl)	5.64	2.45
Safranine	5.45	3.41
Malachite green (oxalate)	7.60	7.52
Crystal violet (Cl)	1.68	13.87
Eosin	44.20	2.18
Erythrosine	11.10	1.87
Fuchsin (basic) (Cl)	0.26	5.93

Staining Bacteria. For general bacteriological work basic stains (*i.e.*, compounds of color bases) are often used, because bacteria react toward stains as though composed principally of nucleic acid, which takes basic dyes most readily.[5, 6] Methylene blue, safranin, crystal violet, basic fuchsin, eosin, etc., are most generally used. All belong to the group of aniline (coal tar) dyes. Any simple aniline dye solution may be applied by flooding the smear with it. Löffler's methylene-blue solution[*] is very widely used and reveals many details of form and structure. Other solutions and colors are also available for special purposes. The dye is allowed to remain in contact with the smear for about one minute and is then washed off with a gentle stream of cool water. The slide is then blotted (*not rubbed*) between two pieces of filter or blotting paper and when dry is ready for examination with the oil immersion lens as previously described.

Now, a simple stain such as Löffler's is of great value for many purposes. But another staining procedure, devised by the Danish scholar Gram, is more valuable because it enables us to differentiate kinds of bacteria, which may be of different species yet of the same general form and size. It is therefore called a *differential stain.*

In addition to Löffler's and Gram's stains, there are certain stains designed to bring out special details such as spores, capsules, flagella and so on. Description of each of these methods will, however, be reserved to the discussion of the structural features of the bacteria to which it applies and to which we shall give some attention in the next chapter.

Gram's Stain.[§] To the smear which, for purposes of discussion, we shall assume to be of a substance containing a variety of bacteria (*e.g.*, saliva) and prepared as described above, crystal violet solution[†] is applied for thirty seconds. This is gently rinsed off and an iodine solution[‡] is applied for thirty seconds. This, in turn, is rinsed off. Ninety-five per cent ethyl (grain) alcohol is applied and renewed

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[*] *Löffler's stain* (Soc. Amer. Bact. "Manual of Methods").[6]

Solution A

Methylene blue	0.3 gm.
Ethyl alcohol (95 per cent)	30.0 ml.

Solution B

KOH (0.01 per cent)	100.0 ml.

Mix solutions A and B. The mixture keeps well.

[§] *Gram's stain* (*Hucker's modification*, Soc. Am. Bact. "Manual of Methods").[6]

[†] Solution A

Crystal violet (85 per cent dye content)	2.0 gm.
Ethyl alcohol (95 per cent)	20.0 ml.

Solution B

Ammonium oxalate	0.8 gm.
Distilled water	80.0 ml.

Dilute solution A about 1 to 5 with distilled water and mix with an equal volume of solution B.

[‡] *Lugol's iodine:*

Iodine	1 gm.
Potassium iodide	2 to 5 gm.
Distilled water	300 ml.

Allow to stand 24 hours for the iodine to dissolve. It may be necessary to add a few more crystals of potassium iodide.

until all but the thickest parts of the smear have ceased to give off dye. (This usually takes from twenty seconds to one minute.)

The *differential* feature of the method is now apparent. Examination with the microscope will reveal the fact that, as Gram found, while many bacteria retain the violet-iodine combination, others will have yielded it entirely to the alcohol and are as colorless and as nearly invisible as before. Those species of bacteria *which retain the stain are called gram-positive.* Those *which yield it to the alcohol are called* gram-negative.

But the staining process is not yet complete. There is still the important final step of applying the *counterstain**—a dye of some contrasting color, usually eosin (red), safranin (red), brilliant green or Bismarck brown. Any one of these dyes colors the gram-negative species and they become as visible as the gram-positive ones, but are readily differentiated by their color.

Thus, by applying Gram's stain, which takes but five or six minutes, we can learn a great deal about any bacterium. We make visible not only the form and size and certain other structural details, but we can also at once assign the organisms which are present in the material being examined, to one of two great artificial groups of bacteria; the gram-negative or gram-positive. As will be seen later, this is very helpful in identification procedures. It should be noted, however, that some organisms are "borderline cases" in respect to Gram's stain, sometimes being positive, other times negative; and sometimes both positive and negative cells are seen in the same culture. As a rule, repeated tests will reveal the true nature of the bacterium. Often slight variations in cultural conditions or staining technic will affect the result. For example, many bacteria are not definitely gram-positive unless cultivated in the presence of at least 5 per cent blood or serum.

As will be seen later the property of gram-positiveness is associated with other properties, especially sensitivity to certain poisonous substances such as sulfadiazine, *basic* dyes like crystal violet, and antibacterial substances of microbial origin, among them penicillin and gramicidin. Gram-negative bacteria on the contrary are markedly sensitive to certain other substances, such as *acid* dyes (sodium azide, *acid* fuchsin, etc.), salts like potassium tellurite and drugs such as sulfaguanidine and sulfasuccidine.

Mechanism of the Gram Stain. If we could learn just *why* some organisms are gram-positive and others gram-negative, we could perhaps clarify questions surrounding the nature and distribution of the nucleus of bacteria, the alterations in Gram staining properties of an

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* *Counterstain:*

Safranin (2.5 per cent solution in 95 per cent alcohol)	25 ml.
Distilled water	75 ml.

organism under different conditions of growth, the structure of the cell wall or cell membrane, and a number of other problems, especially vital questions on bacterial genetics and chemotherapy.

One theory holds that the gram-positive material exists as a sort of outer bark or shell, or perhaps as part of the living exterior portion, and that the underlying protoplasm, forming a sort of core, is gram-negative. Another theory is that the crystal-violet stain and iodine pass separately through the cell membrane, and that they then unite inside the cell to form a compound, the molecules of which are too large to diffuse out again or which is less soluble in the alcohol than either dye or iodine alone. A third idea is that the violet and iodine form an alcohol-insoluble precipitate or coating on the outside of gram-positive cells. A fourth view centers around the isoelectric point of the cell material, relative to the basicity of the dye. Gram-positive organisms have a greater acidity, at least at their surfaces (as measured by isoelectric point), than gram-negative, hence a greater affinity for basic dyes. None of these theories adequately explains the facts.

There seems no doubt that the gram-positive material in bacteria is ribonucleic acid. Bartholomew and Umbreit[7] have shown that it can be removed by soaking the gram-positive cells in sodium choleate. It may be replaced by treating them with magnesium ribonucleate. Normally gram-negative species will not accept the applied coating. The specificity of these reactions is shown by the fact that an enzyme, ribonuclease, will remove the gram-positive character (ribonucleic acid) of the cells very quickly.

The specific character of the Gram stain is further evidenced by the facts that *only* crystal violet will serve in the Gram stain, and only *iodine* will react with it satisfactorily. The essential Gram reaction, therefore, appears to depend on ribonucleic acid in a special combination on the cell surface with crystal violet and iodine.

It appears that the mere presence of ribonucleic acid on the surface of cells is not in itself sufficient to make them gram-positive. It appears to be present in some sort of easily disrupted specific physicochemical combination or state, dependent on the *integrity of the cell.*

This is shown by the fact that *mechanical destruction of the cell,* as by grinding, completely destroys gram-positiveness.

Ziehl-Neelsen Stain. Another differential stain is that of Ziehl-Neelsen. It is used especially for staining tuberculosis bacilli in sputum and is applicable to related organisms having the chemical composition peculiar to these bacilli, *i.e.,* an abundance of waxy material in the cell. By Gram's method such organisms are stained purple but this method does not give as much information about them as the Ziehl-Neelsen or "acid-fast" stain.

In using this stain a smear of the material to be examined is made as usual, dried, and fixed by heat. The smear is then flooded with the solution of carbolfuchsin and heated to 90.0° C., over a steam bath for four minutes. This softens the wax and the dye supposedly penetrates. After washing off the excess dye, the smear is treated for five minutes with cold 95 per cent alcohol containing 5 to 10 per cent hydrochloric acid. Presumably the wax retains the red dye in spite of the acid-alcohol, which removes the color from everything else. If now methylene blue or brilliant green be applied as a counterstain, the tubercle bacilli stand out as bright red bacilli in a blue or green field. The Ziehl-Neelsen stain* is a *differential* stain because it differentiates acid-fast organisms from nonacid-fast ones (Fig. 30).

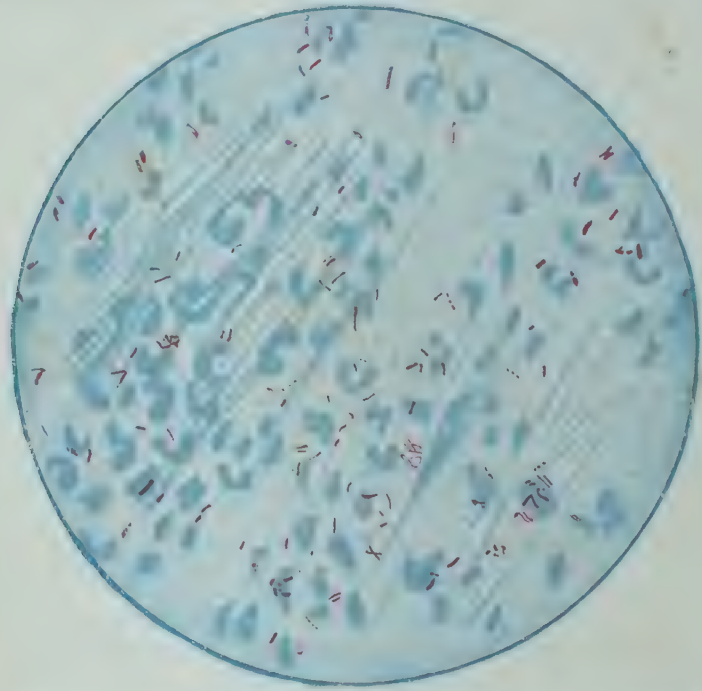

Fig. 30. Tubercle bacilli in sputum. Ziehl-Neelsen; magnified 900 times. The bacilli have been stained red. The mucus and pus cells in the sputum are stained blue for contrast. Note the granular appearance of some of the bacilli, and their arrangement in clusters. The many bacilli pictured here are in a very tiny fraction of a droplet. (Cornet and Meyer.)

Mechanism of the Acid-fast Stain. Acid-fastness, like gram-positiveness, disappears with physical disruption of the cell. It is of interest

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* *Ziehl-Neelsen acid-fast stain (carbolfuchsin):*

Solution A

Basic fuchsin	0.3 gm.
Ethyl alcohol (95 per cent)	10.0 ml.

Solution B

Phenol (melted crystals)	5.0 ml.
Distilled water	95.0 ml.

Mix solutions A and B. The mixture keeps well. A counterstain of Löffler's methylene blue is generally used, although some workers use brilliant green or saturated aqueous solution of picric acid for better contrast.

also that application to acid-fast bacilli of solvents which extract lipids (waxes, etc.) does not remove their acid-fast property. The mere presence of acid-fast waxes, etc., is, therefore, not sufficient to account for this property. Lamanna has presented evidence indicating that acid-fastness is merely a matter of relative solubilities. For example, fuchsin is more soluble in phenol than in water, or alcohol and water. Phenol, in turn, is more soluble in lipids or waxes, such as are present in tubercle bacilli, than in water. In the acid-fast staining procedure, the phenol, with fuchsin in it, leaves the water alcohol of the carbolfuchsin dye mixture and enters the cell lipids in which it is more soluble. Here it remains because it is here more soluble than in the decolorizing agent (acid alcohol) and because the intact cell membrane prevents lipids from leaving the cell to dissolve in the decolorizer. If the cell membrane is broken, the lipids leave the cell and the acid-fast property disappears.

It is of interest to note that if plain water, hot enough (80–90° C.) to dissolve the lipids, be used to wash the stained bacilli, the acid-fast stain is removed.

The appearance of beading in acid-fast stained tubercle bacilli is apparently due to the separation of the acid-fast lipids (when wash water is applied) into globules which arrange themselves like a row of beads in the cell. It has long since been suggested that these beads represent nuclear material or "regenerative granules"—Much granules.[8]

Knaysi regards the cytoplasmic membrane as the seat of the acid-fast property. This would not be inconsistent with Lamanna's theory of solubilities.[9]

More recently Yegian and Vanderlinde have presented strong evidence that after the dye penetrates through the cytoplasmic membrane most of it remains as free dye trapped inside the cell because it cannot diffuse out again. The formation of beads is due to precipitation of dye within the cell.[9a]

Negative Staining. Another method of demonstrating bacteria is by means of "negative staining." This is not really a method of staining bacteria, but of staining the background a solid black, usually with nigrosin.* This fails to penetrate the bacteria at all, and leaves them unstained to appear as light areas in the darkened field (Fig. 31).

* *Dorner's method:*

(*Grease-free* slides *must* be used)

Nigrosin	10 gm.
Water	100 ml.

Boil 30 minutes and add 0.5 ml. formalin. Filter through paper and store in 2 ml. amounts in sterile tubes. Place a loopful of the suspension to be examined on a slide. Immediately add an equal amount of the nigrosin solution, mix, and spread out in a *thin* film. (Thick films will crack and peel.)

Dry in air. *Do not wet the* slide! *Do not heat it!*

Only the outlines of the organism are made apparent by this method, and consequently it has a limited application, being used mainly for such forms as cannot be stained by any of the ordinary methods, especially spirochetes.

Darkfield Method. A method analogous in principle and purpose to the "negative staining" procedure is that of darkfield illumination. The ordinary compound microscope may easily be equipped for this work by substituting a darkfield condenser (purchaseable from microscope manufacturers) in place of the Abbé condenser. In a darkfield condenser an opaque stop is arranged to *prevent* the entrance of any *direct* rays of light from the mirror *straight upward* into the tube of the microscope. All *peripheral* rays are, however, reflected *obliquely* to the *center* of the upper surface of the microscope slide, emerging from the upper surface of the slide at such an angle that they do not enter the objective lens unless some object be present to *reflect* them *upward*. The empty field, therefore, appears dark (Fig. 32). When a fluid containing any particles such as dust, bacteria or spirochetes is placed on the slide at the focal point of these oblique rays, each particle becomes visible as a brightly illuminated speck due to the light reflected upward by it from its surface into the barrel of the microscope (Fig. 33). The remainder of the field appears black, hence the term *darkfield*. Unlike the process of negative staining, the darkfield shows not only outward form but motility, since the organisms, covered with a cover slip, are examined in a moist, *living* state. As outward form and motility are the chief means by which certain important

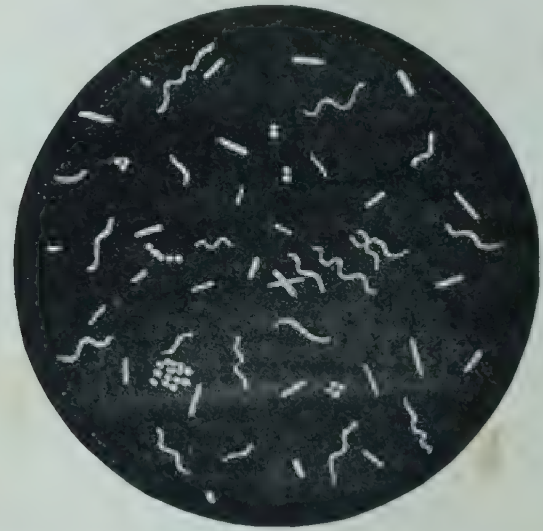
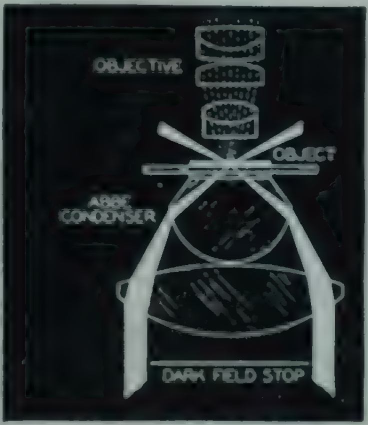
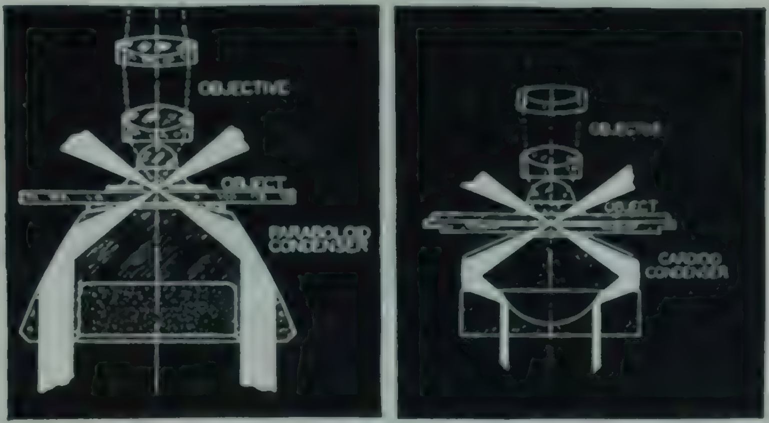


Fig. 31. Appearance of various bacteria as seen when prepared by negative staining method (× 900).

bacteria, especially spirochetes, are distinguished, the method is very valuable in types of work where such species are involved; for example, diagnosis of syphilis.


OBJECTIVE
OBJECT
ABBE CONDENSER
DARK FIELD STOP

Abbé Condenser with
Dark Field Stop


OBJECTIVE
OBJECT
PARABOLOID CONDENSER
OBJECTIVE
OBJECT
CARDIOID CONDENSER

Paraboloid
Condenser

Cardioid
Condenser

Fig. 32. Various forms of condensers for oblique illumination of the darkfield. (Bausch & Lomb Optical Co.)

ELECTRON MICROSCOPY

When Leeuwenhoek showed how to prepare and use lenses to investigate objects so small as to be entirely invisible to the naked eye, and reported his observations of protozoa, bacteria, etc., he was credited with one of the greatest contributions ever made to the advancement of science. As biological investigations continued, however, it became apparent, especially after the discovery of tobacco mosaic virus by Iwanowski in 1892, that there were creatures and objects so small as to make bacteria appear enormous by comparison and which could not be seen even with the most highly developed lens system.

The difficulty lay not with the lens makers but in the fact that the smallest wave length of visible light (blue-violet) is about 4000 A° (A° = Angstrom Unit = 0.000,000,01 cm.). Particles with a diameter of less than one half of this wave length or 2000 A°, or lines separated by a distance less than about 1800 A°, are not clearly visible, or become blurred, because of diffraction effects which cause interference of waves of 4000 A° length. The oil immersion lens and compound microscope generally used by students magnifies only about 900 times. The best that can be expected of ordinary light microscopes is to reveal clearly details larger than 0.000,03 cm. in diameter or somewhat over one half the shortest waves of visible light. Such details are relatively large as compared with many viruses. It is clear that advances in fields of investigation necessitating knowledge of *extremely minute* particles have been definitely restricted by limitations in optical equipment dependent on visible light.

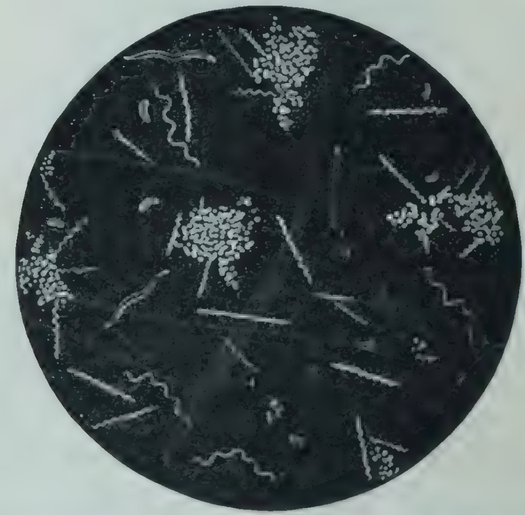


Fig. 33. Darkfield preparation (× 900). This picture shows a group of spirochetes and other bacteria from a lung abscess.

While it has long been known that electromagnetic radiations such as x-rays have very short wave lengths, the preparation of glass lenses or reflectors for such radiations is impossible, so that they cannot be exploited for optical purposes in the ordinary way. Although glass is opaque to electrons, two discoveries in the field of electronics have formed a basis for the use of electrons in microscopy. First, it was observed that electrons are deflected from their line of propagation by magnetic fields and, second, it was found that electrons moving with the speed imparted by 60 KV have a wave length of only about 0.05 A°, or 1/100,000 that of visible light. The latter discovery made plain the possible usefulness of electronic waves in microscopy, while from the former it was clear that circular magnetic fields could be used for "refracting" electron beams, much as a lens does light rays, forming electron images in the same manner that visible light images are formed by lenses.

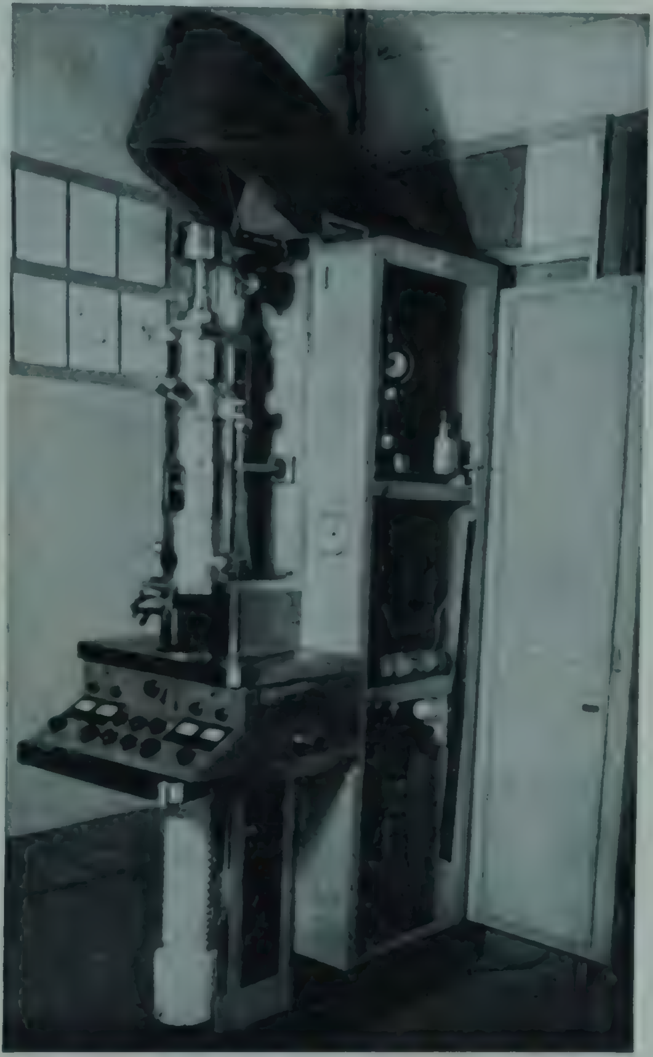


Fig. 34. Advanced universal RCA Electron Microscope. The "heart" of the microscope is the stainless steel column (left) at the top of which is the electron gun. At the bottom of the column, just above the control panel, is the viewing chamber which has three windows so that several people can observe the image without the necessity of looking through port-holes as in the previous design.

OOD TECHNOLOGICAL RESEARCH INS

From these two basic discoveries the modern electron microscope (Fig. 34) has evolved.[10, 11] The units in this instrument are nominally

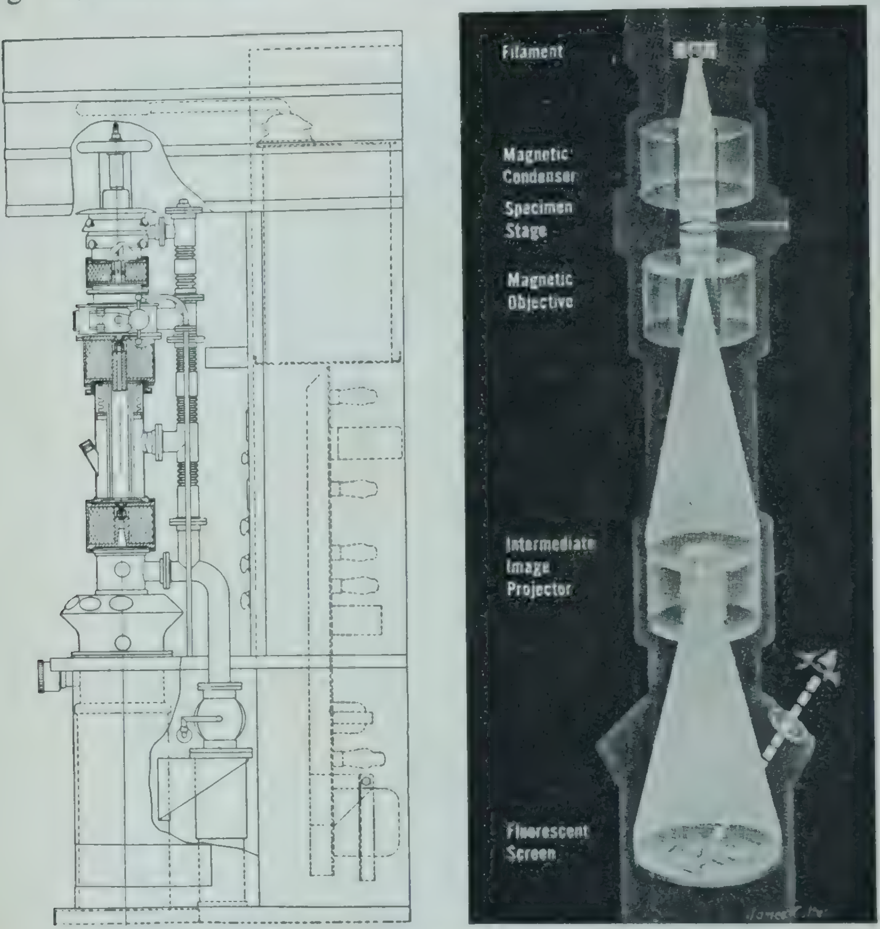


Fig. 35. Simplified sectional view of the RCA Electron Microscope.

Simplified sketch of the electron microscope developed in the RCA Laboratories at Camden, New Jersey. Diagram at right shows how closely analogous the operation of the electron microscope is to the conventional light microscope (right). Suitably shaped magnetic fields take the place of conventional glass lenses. A beam of electrons traveling at high velocity (at voltages of from 30,000 to 100,000) takes the place of ordinary light. The electron rays are converged on the specimen by condenser lens. After passing through the specimen, the objective lens coil forms a first image, enlarged about 100 times. The projection lens coil then magnifies the image again about 250 times, making an overall magnification of 25,000. The final enlarged image can be viewed directly by causing it to strike a fluorescent screen which makes it visible, or it can be made to record the image on a photographic plate for permanent record. The RCA Electron Microscope has such enormous resolving power that the final photograph can be usefully magnified by photographic enlargement up to 100,000 diameters.

analogous with units in an ordinary compound microscope but deal with electron beams rather than light rays. The diagrams in figure 35 show the general arrangement of the focusing systems and other parts of an electron microscope compared with those of a light microscope.[12] The electron source is a tungsten filament at 30–100 KV potential. The electrons enter the magnetic condenser (analogous to an ordinary microscope condenser) and are concentrated on the object. They then pass through the object and enter the axially symmetric magnetic field of the objective magnet where they are focussed, and may be seen by interposing a fluorescent screen, the object being magnified about 100 times. The central (most clearly defined) area of this image is again magnified by an intermediate magnet corresponding to the "eye piece" of a light microscope and the image is projected into a second fluorescent screen or photographic plate corresponding to the eye of the student, with an added magnification of about 250 times (total 25,000). Portions of the photographs may be enlarged 4 times without undue loss of detail, thus giving a picture 100,000 times as large as the object. This degree of magnification is almost inconceivably great. A vague notion of it may be gained by imagining the page on which this is printed to be enlarged to the same degree. It would become a slab of spongy material as thick as a 2-story building is high (to get the effect of the height look down from the 2nd floor), over 9½ miles wide and about 14½ miles long (to get the full effect of these measurements, walk the distance along a hot, dusty road). A good comparison of electron microscopy with ordinary microscopy is seen in Figure 36.

Several details of the operation of the instrument are of interest. As the motion of electrons is impeded by air, the interior of the microscope must be maintained at a vacuum by means of suitable pumps. This necessitates air locks for the insertion and removal of objects and photographic plates. The object mount is manipulated by a lever from outside the instrument which moves the object mount into a small chamber or lock which may be shut off from the main tube of the microscope. An object to be inserted is placed on the mount and moved into the lock which is closed and evacuated by an auxiliary pump before being opened to the main tube of the instrument. To remove the object, the steps are reversed. The lock is closed, air is admitted, the outer door opened and the object removed. The operator can look into the main tube, by means of portholes, and see the images on the fluorescent screens, manipulate the object, make suitable adjustments of field strength of the focusing magnets, alignment, etc. The object is mounted on an extremely thin (0.000001 cm. thick) cellulose film, since glass slides would be opaque to electrons. In newer forms (Fig. 34) of the instrument the details of vacuum manipulation have been much simplified, and other mechanical improvements installed.

A convenient development is the small console model (Fig. 37). Many exceedingly interesting studies have been made in the fields of chemistry, physics and biology with results of incalculable value. Crystal structure, colloids, organic molecules, film and filament structures, details of bacterial and other cell structure and many other

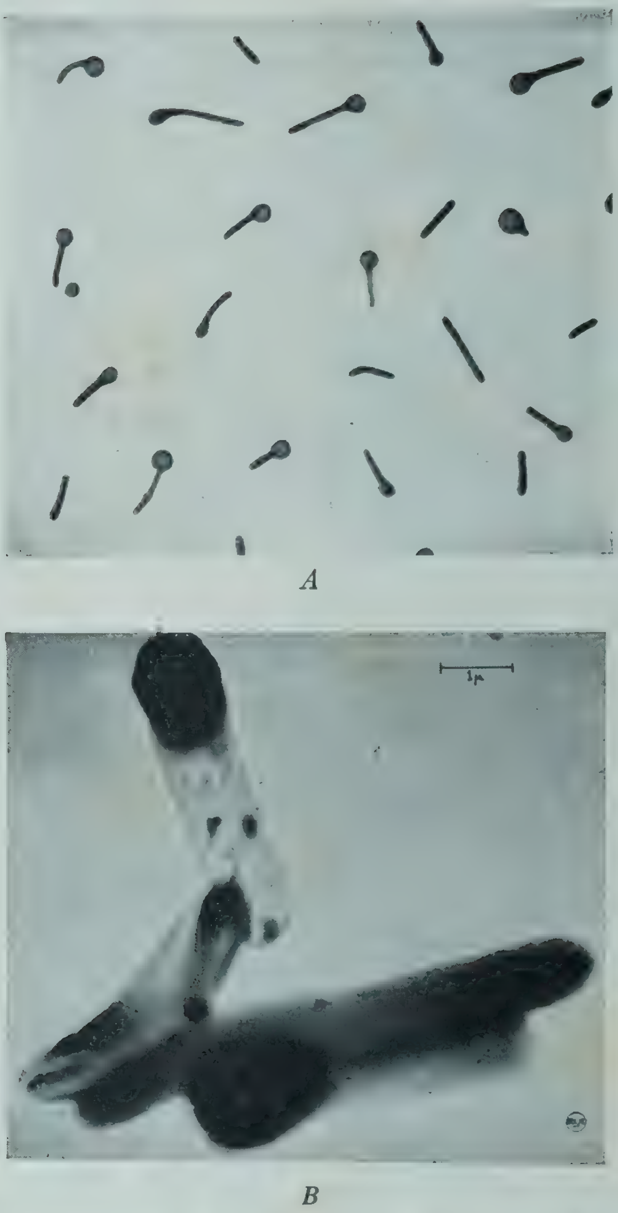


Fig. 36. Two pictures of the lockjaw organism (*Clostridium tetani*) as seen magnified × 2000 by ordinary microscope (*A*) and magnified × 14,000 by electron microscopy in *B*. The terminal spores are seen as large dark bodies. The smaller dark bodies in the enlarged cells may be artifacts, parts of the cell structure, or folds in the cell membrane. (*A*, Ford, Textbook of Bacteriology, W. B. Saunders Co.; *B*, S. Mudd and T. F. Anderson, J.A.M.A., 1944.)

matters hitherto hidden from us are now subject to our scrutiny. Several reproductions of electronographs (pictures made with electron microscopes) are found farther on in this book. Under conditions of electron microscopy living organisms cannot survive and physiological processes in live cells cannot be studied by this means at present.

A word of caution may be spoken in closing our discussion. The "objects" seen in photographs of electronic images are merely shadows, much like shadows on an x-ray plate, and depend for visibility only on degree of opacity, to *electrons*, of parts of the object. They may represent actual structures, or mere thickenings of the object, or artifacts. In any case, our interpretations of them should be extremely guarded until much more is known of electronic microscopy and its pitfalls.

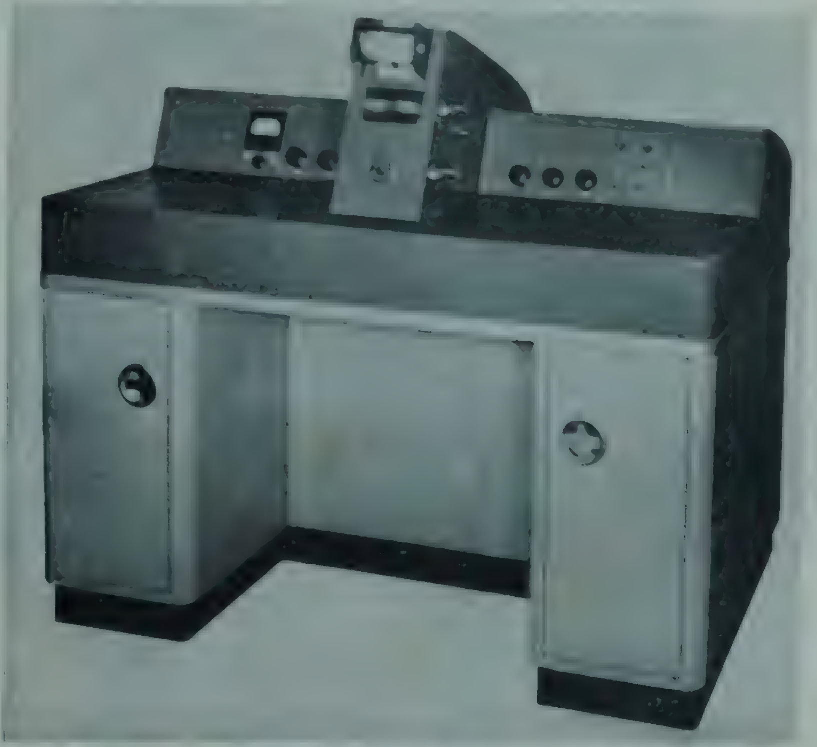
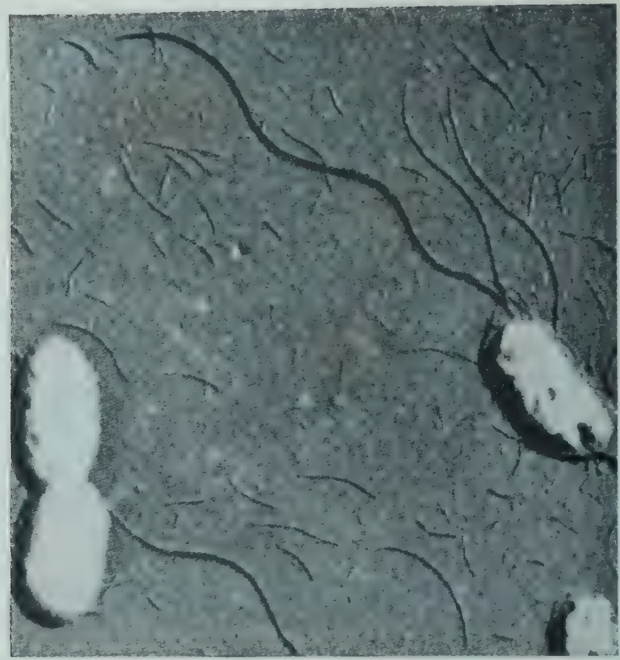
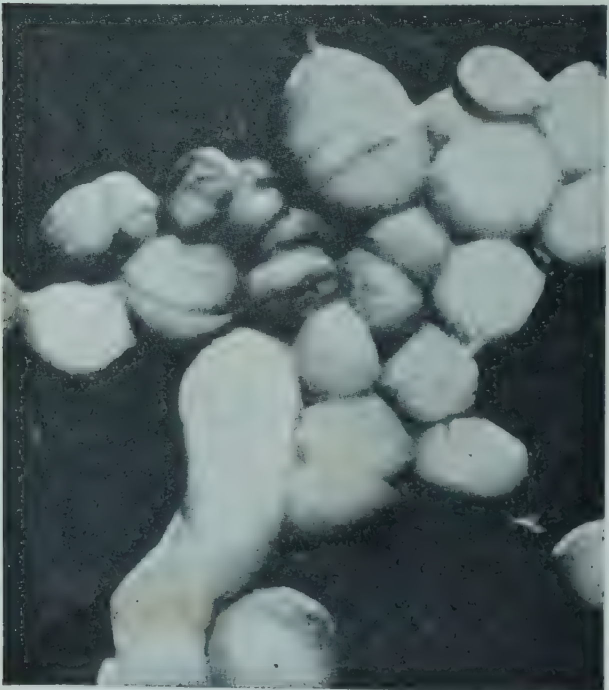


Fig. 37. The famed electron microscope, in console desk form, which makes this powerful explorer of the sub-microscopic world available to smaller laboratories, schools, hospitals, and factories. Opening unexplored regions to a host of research scientists, this new console electron microscope is capable of magnifying minute particles of matter up to 100,000 times.

Because of the minuteness of many biological structures, such as viruses, they contain little or no matter which obstructs the electrons and so cast no shadows when electrons are passed through them. Thus they are entirely transparent to the beams. In order to visualize



A

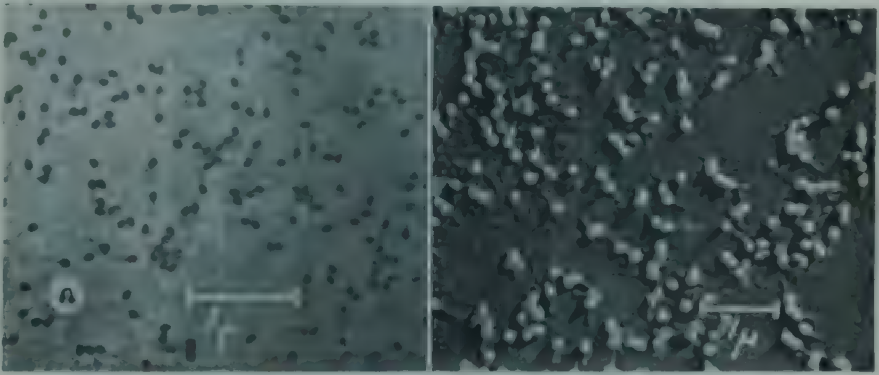


B

Fig. 38. Electron shadow micrographs of: *A, Salmonella typhosa,* showing flagella (Courtesy Dr. Robley C. Williams, Univ. of Michigan); *B*, an old culture of *Staph. aureus.* In *B* many interesting details of cell morphology are seen

detail, it is necessary to produce contrasts in the object. This may be done in one way by selective staining (as with phosphotungstic acid) so that some parts of the object are made more "opaque" to the electrons than others.

One of the most valuable of the new techniques for the microbiologist is that called electron shadow-micrography, developed by Williams and Wyckoff. This is best described by the inventors as "depositing an exceedingly thin (ca. 7 $\mu\mu$) layer of metal (chromium or gold) obliquely (in vacuo) on the preparation . . . High points on the surface intercept more than an average number of the condensing atoms (of metal) and at the same time shield areas immediately behind them. These shielded areas, devoid of deposited metal, appear as "shadows" of the elevated regions responsible for them. Just as an observer . . . in a plane sees many details of a landscape at sunrise, or sunset, which would be invisible to him at noon, so metal shadow-casting brings out much detail that is not to be seen in an unshadowed (or in a vertically coated) preparation." The advantages in this technique are seen in figure 38. The method applied to viruses is wonderfully revealing (Fig. 39A and B). Many other interesting "shadowed" pictures have been made, showing, for example, the details of structure and size of Lansing poliomyelitis virus, tobacco mosaic virus and several species of bacteria.



A *B*

Fig. 39. Electronographs of a virus (bacteriophage). *A*, without metal shadowing; *B*, with metal shadowing. (Courtesy of Dr. D. Gordon Sharp, Duke University.)

Other useful techniques for electron micrography include the

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that would be missed by ordinary electron micrography. The significance of these details remains to be fully explained. (Williams, R. C., and Wyckoff, R. W. G., in **Proc. Soc. Exper. Biol. and Med., Vol. 59**).

preparation of plastic replicas or "casts" of the surfaces of bacteria, metals, ceramics, etc. The surface of the replica is then "shadowed" so as to reveal details even so small as molecules. The use of metal coating and plastic replicas, in conjunction with frozen-dried preparations yields pictures of cells which are very little distorted or shrunken.[13, 14, 15, 16, 17, 18]

Two important new developments in electron microscopy have permitted the production of greatly improved pictures of microorganisms. One of these improvements consists of a two-component objective giving maximum contrast from unstained biological specimens.[29] The other improvement involves cultivating organisms on a very thin collodion membrane deposited on the surface of a solid or liquid nutrient medium.[30] The air immediately above the organisms is kept fairly dry. Motility of organisms is thus held to a minimum. The growth on the membrane may be lifted from the medium and examined in the electron microscope after desiccation, thus introducing very few artifacts (Fig. 39a).

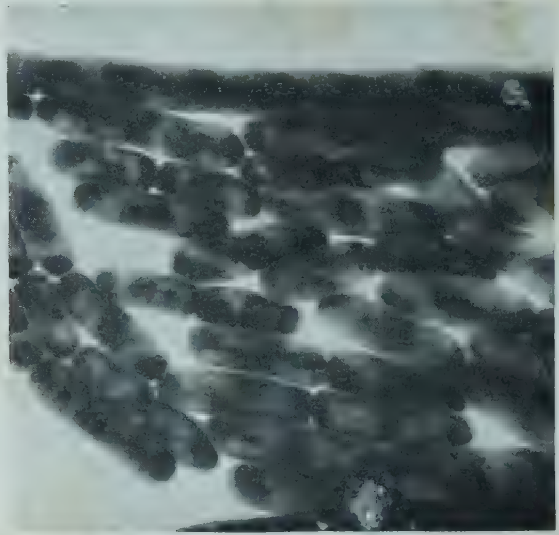


Fig. 39a. Exceptionally clear picture of very young cells of *E. coli* (a common, harmless, intestinal bacterium) examined by the improved lens and mounting technic. This shows density variations inside the cells which are variously interpreted by different observers. It is evident that the cell structure is complex. (Reduced ⅓ from photo representing a magnification of 7600.) Reproduced from original print published by Hillier, J., Mudd, S. and Smith, A. G., J. Bact., 1949.

In 1948 it was reported that *proton* microscopes are available which can magnify up to 600,000 diameters. Protons have a much shorter wave length than electrons. The mechanical aspects of such instruments will be somewhat bewildering. Protonographs should reveal molecular structure (Fig. 40).

FLUORESCENCE MICROSCOPY

By fluorescence is meant the property possessed by certain substances of reflecting rays having a wave length different from that of the incident rays. Thus, substances having a certain color by ordinary light appear of a totally different color by ultraviolet light. Objects invisible when "illuminated" only by ultraviolet light may become brilliantly luminous if painted with a fluorescent substance such as quinine sulfate (fluoresces violet in ultraviolet light) or the dye auramine (fluoresces brilliant, luminous yellow in ultraviolet rays). By staining bacteria with a fluorescent dye and observing them in a field illuminated only with ultraviolet light, they can be made visible as brilliant, luminous objects readily seen in a black field and differentiated from nonfluorescent objects. This principle has been used for many years in microscopy.[19, 20]

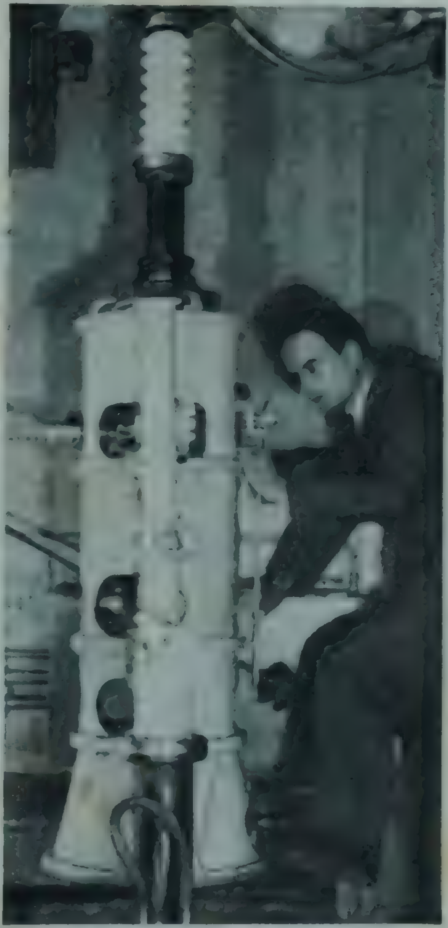


Fig. 40. World's first proton microscope has been built by Frenchman Claude Magnan. (Science Illustrated, Vol. 3)

One adaptation has been in the study of tuberculosis. The fluorescent dye, auramine, has a strong affinity for wax-like substances in tubercle bacilli. When the bacilli are stained with auramine, and examined in the dark by ultraviolet light, they fluoresce with a luminous yellow light.[21, 22]

For light a carbon arc or a low-voltage, high-ampere, concentrated-filament lamp is to be preferred. The glass mirror of the microscope is covered with a polished aluminum mirror since the silver and glass would absorb too much of the ultraviolet light. A blue, ultraviolet-transmitting filter is placed over the lamp to withhold most of the visible light and a fully-complementary yellow filter is placed in the ocular of the microscope (Fig. 41) to permit the passage of fluorescent light while withholding ultraviolet rays which would injure the eye. If the filters are properly complementary no visible light will reach the eye and the field will appear black unless some fluorescent object be placed in it.

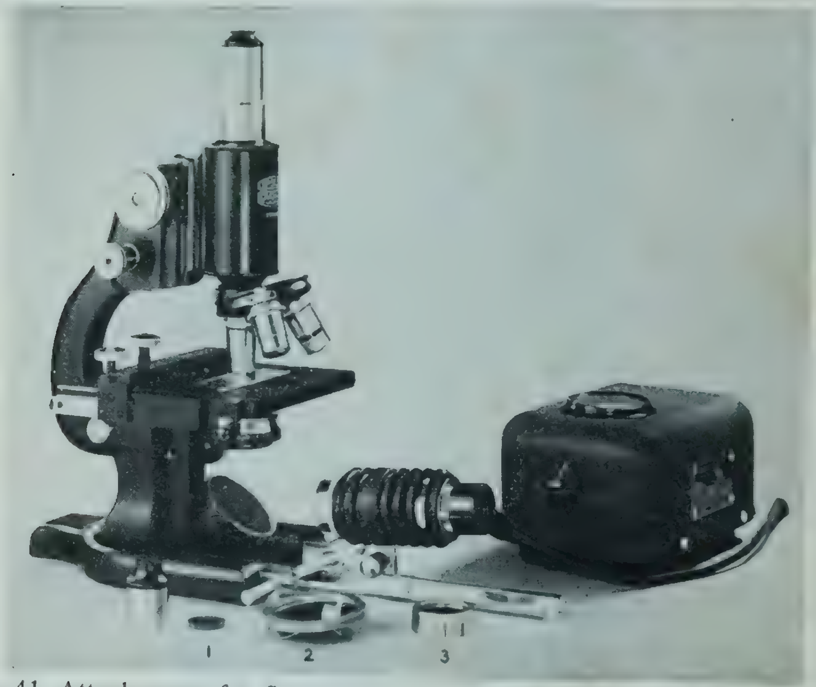


Fig. 41. Attachments for fluorescent demonstration of acid-fast organisms. 1, yellow filter to go into ocular. 2, front surfaced, aluminized mirror fits over microscope mirror. 3, blue, ultraviolet transmitting filter for lamp. (Richards and Miller, Amer. Jour. Clin. Path., 1941, *11*:1.)

PHASE MICROSCOPY

One of the most important new developments in the field of microscopy is the phase microscope. This instrument differs from the ordinary microscope only in that it has a diaphragm with an annular or ring-shaped opening, instead of the usual opening, at the focal plane of the condenser, and a specially devised diffraction plate inserted at a certain focal plane in the objective. The effects of these arrangements are complex, but are based on refraction, absorption, and retardation of waves, and on wave interference.

The student will recall from his studies in physics, that light waves (*B*, Fig. 42) passing through material of greater density than air (*C*, Fig. 42) are slowed, with respect to waves not passing through such material (*A*, Fig. 42). The wave *B* traverses a greater optical path (thickness × refractive index) than *A*. The eye does not detect the difference in phase of waves *A* and *B* but the difference may be made visible by a suitable diffraction plate in the objective, due to interference of the waves in different phase. For example, if a part of the object introduces a slowing of the light of, say 0.25 wave length ($0.25\Delta = 90°$) and this change is reinforced by an additional 90° in the diffraction plate, then that light is 180° out of phase with light passing outside the specified portion of the object, and this portion therefore appears completely dark. Other parts of the object may produce retardation of light in lesser degrees, and these portions will appear as lighter or darker; *i.e.*, in contrast to each other and to the background. In addition, if a portion of the object (*E*, Fig. 42) *absorbs* part of the light (*D*, Fig. 42) the wave *amplitude* or light *intensity* is decreased. Such differences between *intensities* (as between *D*, and *B* or *A*) are visible to the eye. Wave interference is seen in the relations of S to R, Fig. 42.

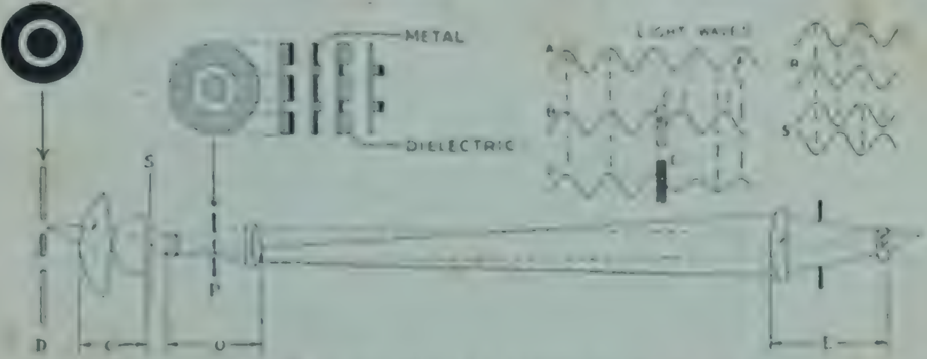


Fig. 42. The annular substage shield is shown at D. C is the ordinary condenser system; S, the stage; O, the objective assembly; P, the special diffraction plate in the objective. The solid and dotted lines show direct and refracted rays of light, respectively. Types of diffraction plate are seen above plate P. The dark zones are metal, which control wave amplitude (transmission), the shaded areas are dielectric, which control all phase differences. (Bennett, A. M., American Optical Co., Scientific Instrument Division, Buffalo, N. Y.)

The diffraction plate in the phase microscope integrates phase and amplitude differences so that portions of the object having different optical paths, and different absorbing properties, are clearly differentiated in terms of brightness or darkness. Thus, with maximum light and resolution, living organisms are seen in sharp outline, with structural features (internal and external) in remarkable detail while by means of ordinary microscopes these differences and contrasts, unless very large (which is rarely the case in microbiology) are imperceptible.

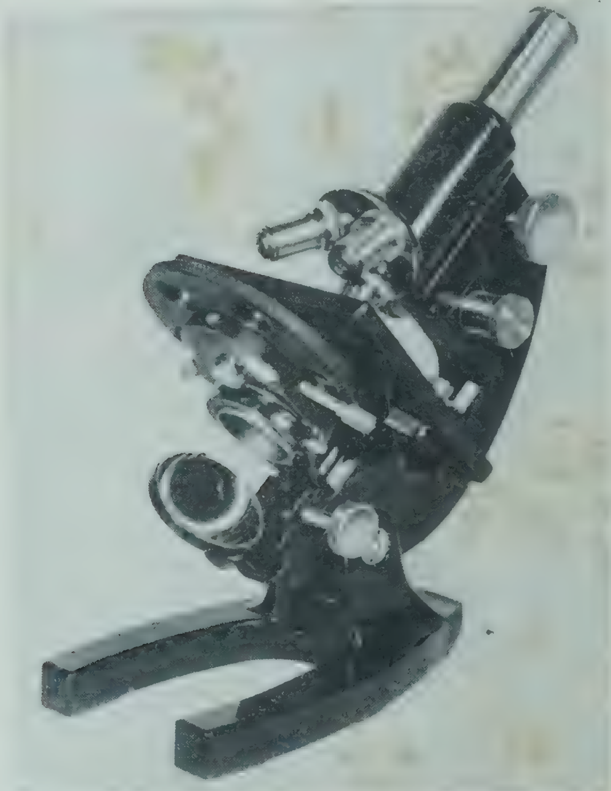


Fig. 43. Phase Microscope. (Bausch and Lomb Optical Co.)

In the phase microscope, light passes through the substage annulus and is brought to a focus at a point within the objective. At this focal point a suitable diffraction plate is placed. On the plate, which is made of glass, a minute, bright, image of the annulus is formed. The ring-shaped area on the plate corresponding exactly to this image is spoken of as the *conjugate area;* all else (center and periphery) is called the *complementary area.* The special features of the plate are that films of phase-altering material, or of light-absorbing dielectric material, or both, are placed over the conjugate or complementary areas in various arrangements. These films on the plate are the part of the apparatus that change phase differences to amplitude differences, and integrate all amplitude differences into an image of great contrast. The exact mechanism of this effect is beyond the scope of this book but may be found in the literature cited.

Plates with various arrangements of metallic and dielectric material are designated according to the effect of the plates on the deviated and undeviated rays. For plates which reduce the *intensity* of the undeviated rays (conjugate area) in relation to that of the scattered rays (complementary area), the designation A is used; for plates having the reverse effect, the letter B. For plates increasing the *phase* of the undeviated rays relative to that of the deviated, (dielectric material on the ring only) a + sign is placed after the letter; for plates with the reverse effect, (dielectric material on complementary area) a — sign is used.

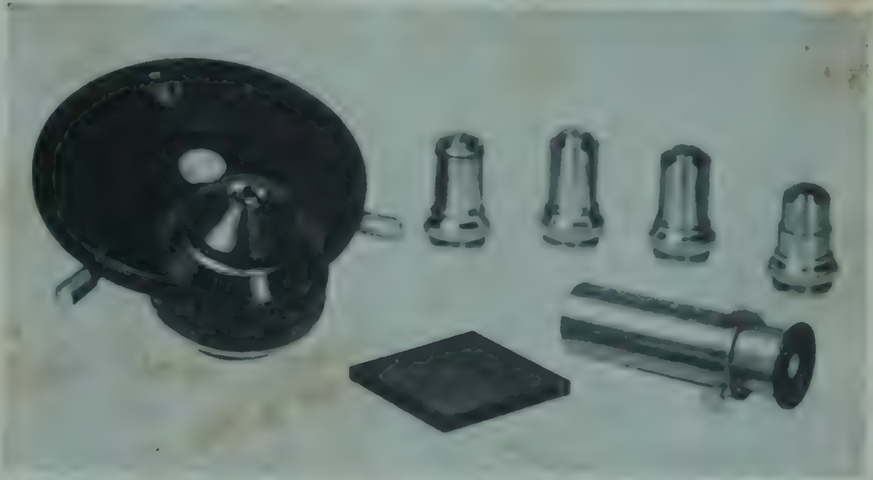


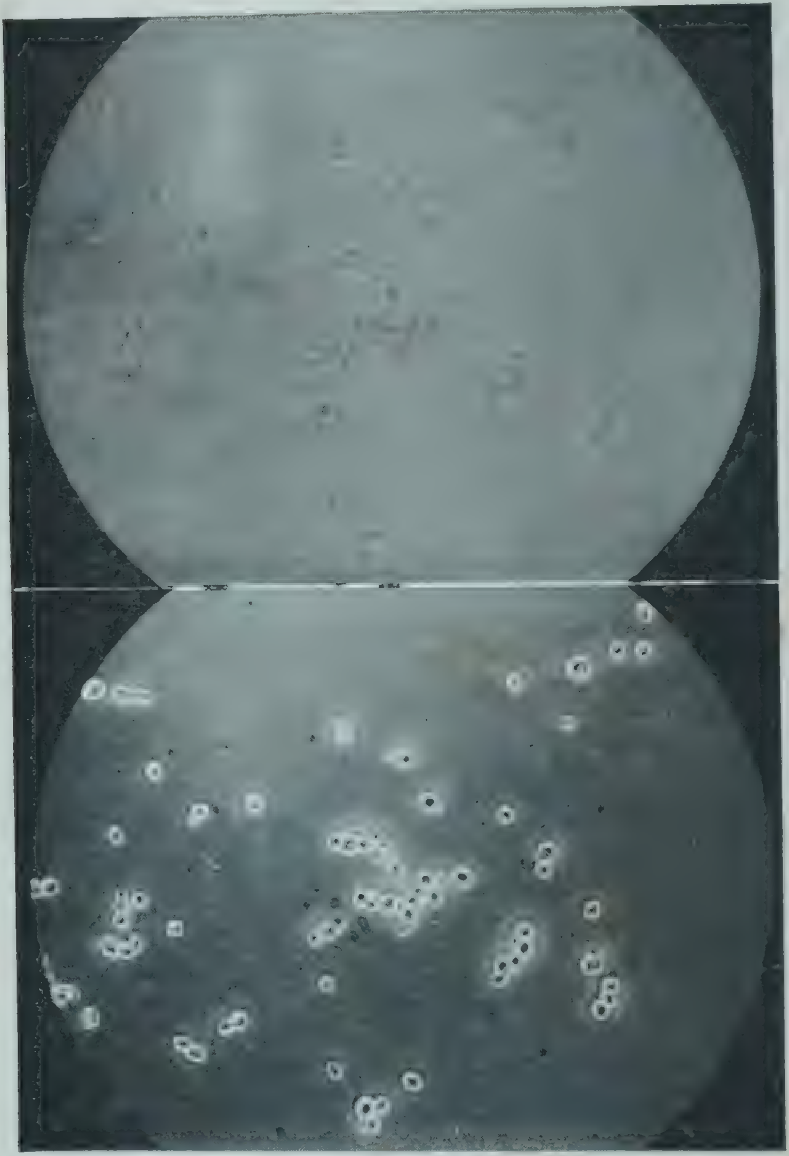
Fig. 44. Phase contrast microscope accessories. (Bausch and Lomb Optical Co.)

Plates are usually described by giving numerical values to the letter and to the + or — sign; for example, in a plate in which the intensity of the undeviated rays is 0.25 that of the deviated, the letter A is preceded by the fraction 0.25. If the plate, because of the dielectric film, also increases the phase of the deviated rays by 0.20 wave length more than that of the undeviated, the — sign is followed by 0.20. Such a plate is marked 0.25 A – 0.20.

If the object is bright and the field darker, the relation is called "bright contrast." The reverse is "dark contrast." These types of contrast may be produced by using different types of plate.

In practice, the image of the substage annulus must be sharply focused on the plate. This may be done by moving the substage diaphragm with the open ring up or down. Also, the plate in the objective must be centered on the image of the annulus. This may be done with a special telescope temporarily used in place of the ordinary eyepiece. In some forms of the apparatus only one objective plate is used with several sizes of annulus; in others, there is one annulus with several forms of plate. Each system has advantages and disadvantages. An ordinary microscope may easily be adapted for use with phase contrast. A special holder is made for the annular

A



B

Fig. 45. Yeast. *A*, Bright field. *B*, Phase contrast. Budding cells from a three-days old culture. In phase contrast, note the dark areas which represent food vacuoles and cell granules, such as glycogen. (Bausch and Lomb Optical Co.)

diaphragm, and special plates, or objectives with plates, are provided. A telescope for centering the plates is also available. The phase objectives can also be used for ordinary work by using an ordinary diaphragm (Figs. 43 and 44).

By means of these adjustable arrangements in the phase microscope, seemingly greater resolution is possible and it is quite feasible to see very small objects, for example, the flagella of living vibrios in motion, and certain viruses. Variations in the direction and intensity of illumination give the most beautiful appearances of darkfield, although the light actually passes through the objects and is not reflected from their surfaces as it is in true darkfield microscopy.

Phase microscopy promises much that is at present impossible by any other means of observation. The method will undoubtedly become available to many laboratories as the cost is less than that of electron microscopes at present.[23-28]

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Chapter 4

MORPHOLOGY AND STRUCTURE OF BACTERIA

Morphological Types of Bacteria. As soon as microscopes and microscopy were sufficiently well developed to permit detailed studies of bacteria, it was found that several forms of these organisms could be distinguished (Fig. 23). It is now clear that three main forms may be differentiated, the spherical, the cylindrical,[1] or rod-like, and the spiral or helicoidal.

Spherical bacteria are called cocci (singular=*coccus*) and these are classified into several groups, mainly on the basis of the manner in which they cling together after fission. First, there are *diplococci*, which remain in pairs. Obviously these divide in one plane. Then there are the *streptococci* which, like the diplococci, divide in one plane but which cling together in chains, looking much like strings of beads. Other cocci divide irregularly in two or three planes, and cling together in masses shaped like bunches of grapes. These are called *staphylococci* or *micrococci*. Still others divide regularly in three planes at right angles to each other, forming cubical groups. These are named *sarcinae*. (Latin for packet.)

The spherical form of cocci is often distorted by various influences, so that oval, elliptical, conical and other modifications are frequently seen.

Cylindrical or rod-like bacteria are called bacilli (singular=*bacillus*) which is Latin for stick or staff. These are straight, cigarette-shaped or sausage-shaped organisms. The cell wall appears to be a fairly rigid tube or sac, since growth occurs mainly lengthwise, and variations in size affect mainly the long axis. Sometimes bacilli are so short that the length is no greater than their diameter. These are often referred to as cocco-bacilli. It is difficult to distinguish them from cocci. However, in *pure* cultures this is not such a great problem, since a few of the cells are usually long enough to reveal the bacillary nature of the culture.

Helicoidal or spiral bacteria are shaped like a coiled wire spring. Two main groups may be recognized: the *flexible* spiral bacteria (classified as the order *Spirochaetales* and commonly called spirochaetes), and the *rigid* spiral bacteria (classified in the family *Spirillaceae*). A subgroup of the latter comprises helicoidal forms which are so short as to consist of only part of a spiral turn; in fact they are shaped much like a slightly twisted comma. These are grouped in the genus *Vibrio* of which *Vibrio comma* (cause of Asiatic cholera) is an important member.

The relations of the morphological types are shown in the following outline:*

1. *Coccus* forms (spherical or derived from spheres)
    - *Diplococcus* (pairs)
    - *Streptococcus* (chains)
    - *Staphylococcus* (irregular clusters)
    - *Sarcina* (cubical packets)
2. *Bacillus* or rod forms (straight, rod-like or cylindrical).
3. Helicoidal forms
    - *Spirochaeta* (flexible; several complete spiral turns. Order Spirochaetales)
    - *Spirillum* (rigid;1 or more spiral turns)
    - *Vibrio* (rigid; less than 1 spiral turn)

As bacteria grow they are subject to many influences which affect their size and shape, much as various factors affect the size and shape of potatoes or peppers. Age also plays its role.[2]

For example, cells enlarge as they continue to grow, or to synthesize cell substance. Now, the volume of the cell increases many times more rapidly than the surface.† Yet foods for the inner parts must pass through the cell surface since there is no mouth. At a critical point in the ratio of volume (which consumes) to surface (which supplies) the favorable initial relationship between surface and content must be renewed. This is accomplished by fission, which increases the ratio of surface to volume and results in the formation of two small daughter cells. These grow and divide and so the process continues.

In a discussion of the internal structure and physiology of cells later on in this book we shall see the biological importance of surfaces and the fact that division of any mass of substance into small particles greatly increases the surface of that substance. A great many biochemical phenomena result from reactions governed by surface forces, such as surface tension and osmotic pressure. These all affect the size and shape and arrangement of cells.

Referring to the straight, rod-shaped, cylindrical forms, it should be pointed out that the term "bacterium" was originally employed for all. However, when endospores‡ were discovered by Cohn in 1875 in the rod-like species of common aerobic bacterium now known as *Bacillus subtilis*, he differentiated these sporulating organisms from the other cylindrical bacteria and adopted the term "*Bacillus*" as a genus name. For this reason, the term "bacillus" should be employed only for aerobic rod-shaped species which produce true endospores. The term "bacterium" is applicable to rod-shaped species which fail to produce these bodies. In general, the terms "bacillus" and "bacterium" are used interchangeably for all rod-shaped cylindrical forms. The term "bacterium" is also used in a general sense to mean any organism belonging to the class Schizomycetes.

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* Terms in italics are titles of genera named for the morphology of the organisms.

† The volume of a cylinder increases as the square of its radius (Xr^2 x alt.); the volume of a sphere increases as the cube of its diameter (diam.^{3} x 0.5236).

‡ Endospores are oval, refractile protoplasmic bodies which develop inside of certain kinds of bacterial cells. Only one is formed in each cell. It represents a dormant stage which is quite resistant to drying, heat, disinfectants, etc., and enables bacteria to survive conditions unfavorable to growth. Spores will be discussed more fully later.

Size of Bacteria. The drawings and reports by Leeuwenhoek (Figs. 1 and 4), the first to observe and describe bacteria (*circa* 1700), gave men an idea of the size and form of certain common types of these organisms.

Their minute size may be emphasized by various comparisons. For example, it is estimated that a cubic inch would hold nine trillion (9,000,000,000,000) cells of a medium sized bacillus (*Salmonella typhosa*); while four hundred million (400,000,000) would occupy only the volume of a "granule" of granulated sugar. It is common practice to magnify bacteria nine hundred to one thousand diameters with microscopes. They may then look no bigger than a period on this page, or an exclamation point (!). A man magnified to the same degree would be over a mile high and 500 yards wide.

The size of bacteria ranges from a diameter of about 0.5 micron* (0.0005 mm.) and a length, in the cylindrical forms, sometimes as small as 1 micron (0.001 mm.). Lengths of 200 to 500 microns (0.2 to 0.5 mm.) are not unknown, however, in certain species.

Bacterial Anatomy. The physiological activities of bacteria are less complex than those of more highly evolved creatures. They do not walk, they do not breathe, they do not write, see, hear, smell, chew, or talk. In fact, they do little else than absorb dissolved food by diffusion, secrete wastes and enzymes and, by virtue of the colloidal make-up of their protoplasm, carry on integrated systems of chemical reactions which result in release of energy and synthesis of the cell substance, the latter including fission as the cells enlarge. These functions require only a little colloidal, jelly-like protoplasm and a cell membrane. Some species also swim by means of flagella. A few form spores. There is no obvious sexuality. Therefore, the anatomy of bacteria in general is simple, as compared with that of a rosebush or a flea.

Surface Structures. Under this heading we may consider 5 divisions, proceeding from exterior inward: (1) flagella, (2) capsule or slime layer, (3) cell wall, (4) cytoplasmic membrane. (5) Inside of the cytoplasmic membrane is the living cell content proper—the cytoplasm and nuclear structures (if any). These will be discussed in their turn.

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* The micron (μ) is the unit of length used in bacteriology. It is $\frac{1}{1000}$ of a millimeter or $\frac{1}{25{,}400}$ of an inch.

Flagella. Flagella, according to the traditional view, are long, very fine[3] threads of protoplasm attached to the cell in various locations, the number and position being determined to some extent by the species (see Fig. 46).[4] Whether flagella originate from the cytoplasm or some outer structure is difficult to determine. According to Knaysi,[3, 5] it is the former. The mechanism by which they are activated is not clear. According to most views, flagella move the bacteria by spiral waves along the length of the flagella (Fig. 47).

Not all bacteria possess flagella. Aflagellate species are non-motile, with a few special exceptions. The power to move by means of flagella, like the power of spore formation, appears to have developed mainly with elongation of form. Very few (if any) spherical bacteria have flagella or form spores.

According to Pijper, flagella are derived from the slimy capsule which is conceived of as being composed of long-chain carbohydrate molecules and of being, therefore, viscous and fibrillar in structure.[6, 7] Pijper regards the flagella as inert. The cell moves because it is helicoidal in form and exhibits undulatory and gyratory motion. Tangles of the capsular fibrils derive their form and motion passively from the motion of the cell, much as ribbons will flutter in a serpentine manner if attached to a rapidly moving automobile. This is not in agreement with observations by means of the phase microscope which show clearly that flagella may move actively even though the cell to which they are attached is entangled motionless in a mass of debris. If Pijper's views are confirmed, many of our ideas regarding the shape and motility of bacteria, as well as their antigenic and biochemical structure and taxonomic relationships must be completely revised. It is of course possible that some bacteria move by the traditional mode of flagellar action and that others are as Pijper describes them.[8] As stated by Dubos ". . . much remains to be learned of the occurrence and importance of flagellation in the bacterial world."[9] (Figures 48, 49, 50.)

According to Conn and Elrod, "flagella must still be regarded as having the significance attached to them in the past." The best evidence is in support of this view.[9a]

With reservations in respect to the very stimulating statements of Pijper, we may note that there are some nonflagellate species which move slowly by creeping, much as do some algae. Some of the spirochetes appear to move without flagella but this may be because flagella have not been demonstrated. Probably they move because they are spiral and contractile.

BACTERIAL MOTILITY. Whatever the mechanism of bacterial motility bacteria can move only in fluids. They cannot leave a fluid surface. They may pass through the air, but not of their own volition. It is only as passengers or riders on particles of dust in the air, as Spallanzani, Pasteur and Lister demonstrated, or on objects like pencils, eating utensils, books, etc., or in droplets such as those given off by a person when sneezing or coughing, or in streams of water, that they can travel great distances.

Bacterial motility is of a very primitive but useful type. The organisms apparently cannot control their speed, which seems always to be at a maximum for a given individual. Whether they can voluntarily change their direction of travel as do paramecia is open to question.

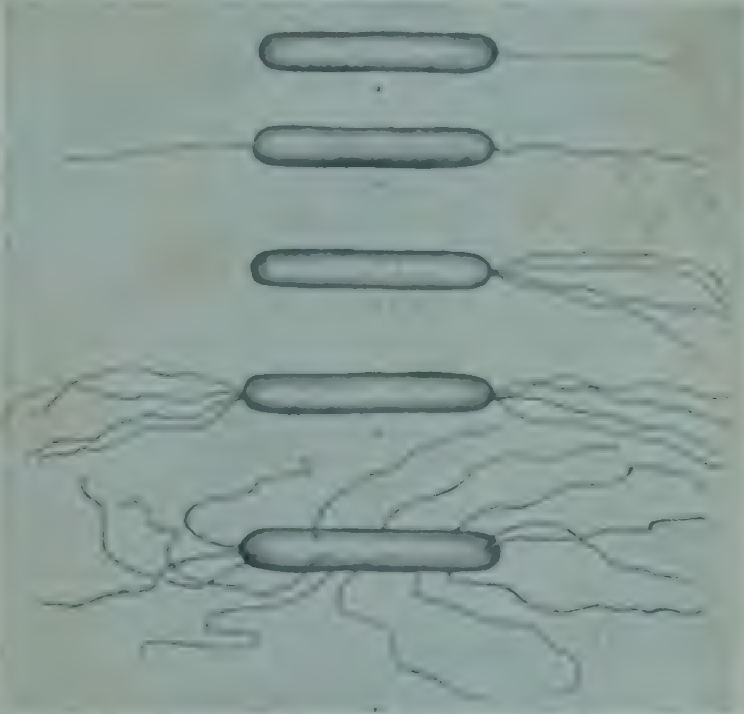

Fig. 46. Types of flagellation: *a*, Monotrichate; *b*, amphitrichate; *c* and *d*, lophotrichate; *e*, peritrichate.

Their speed is sometimes very great when measured by their size. Some of them cover a distance equal to hundreds of times their length in a second. An automobile would have to speed at a thousand miles an hour to accomplish the same thing.

DEMONSTRATION OF MOTILITY. The *motility* is easily visible upon direct observation of the bacteria in a droplet (hanging drop) of the fluid in which they are living. In any culture of bacteria, especially old cultures, motile cells may be difficult to find among thousands of dead or senescent cells. Young cultures should always be used. In cultures acidified by fermentation, bacteria lose their motility. Strict anaerobes lose motility on contact with air.

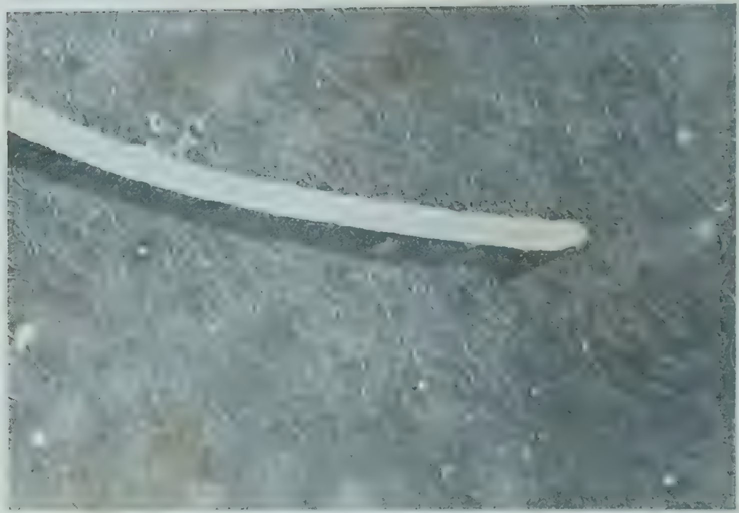

Fig. 47. Electronograph of *Proteus sp.*, prepared by a shadowing technique. This picture shows flagella with exceptional clarity. (Photograph courtesy of Dr. C. F. Robinow and Dr. James Hillier of R.C.A.)

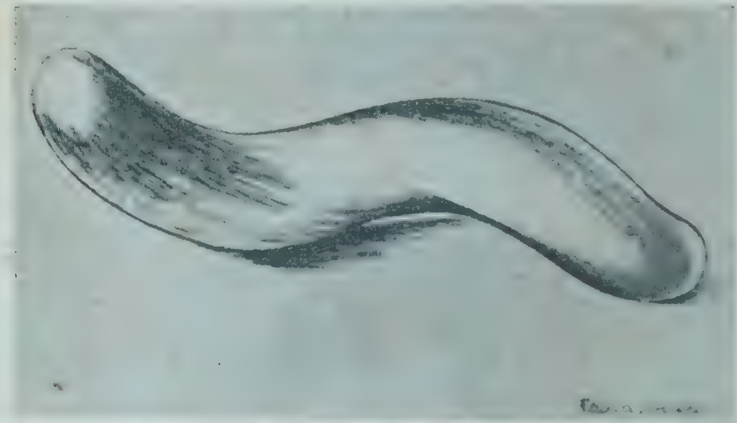

Fig. 48. Perspective drawing of model of motile bacterium, showing spiral shape. (Pijper, Adrianus, in J. Path. and Bact., vol. 58.)

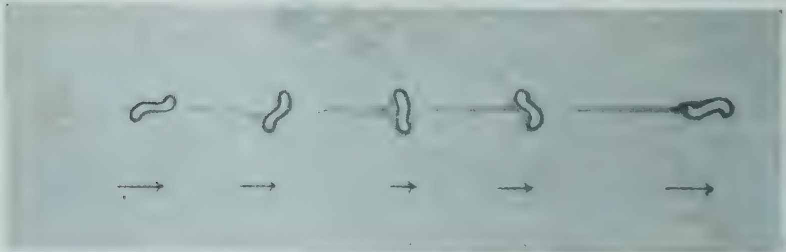

Fig. 49. Diagram of motile bacterium turning half a somersault: the tail remains in place. (Pijper, Adrianus, in J. Path. and Bact., vol. 58.)

It is necessary to distinguish carefully between true motility and brownian movement. Truly motile bacteria progress definitely and continuously in a given direction. Brownian movement is a purposeless oscillation within a very limited area. It is due to molecular forces entirely external to the bacteria. Unless the bacteria are fairly active, brownian movement is sometimes rather difficult to distinguish from true motility.

DEMONSTRATION OF FLAGELLA. The demonstration of bacterial flagella is not so easy as the demonstration of motility. In practice it has been customary to regard motility as *prima facie* evidence of the presence of flagella. This is generally safe evidence when dealing with true bacteria (Eubacteriales) but in at least three other orders of the class Schizomycetes (Spirochaetales, Myxobacteriales, and Chlamydobacteriales) motility is accomplished without the aid of any visible means of locomotion and is not fully explained. Electron microscope observations by Mudd, Povitsky, and others show what seem to be flagella on spirochetes of syphilis (see Fig. 224).

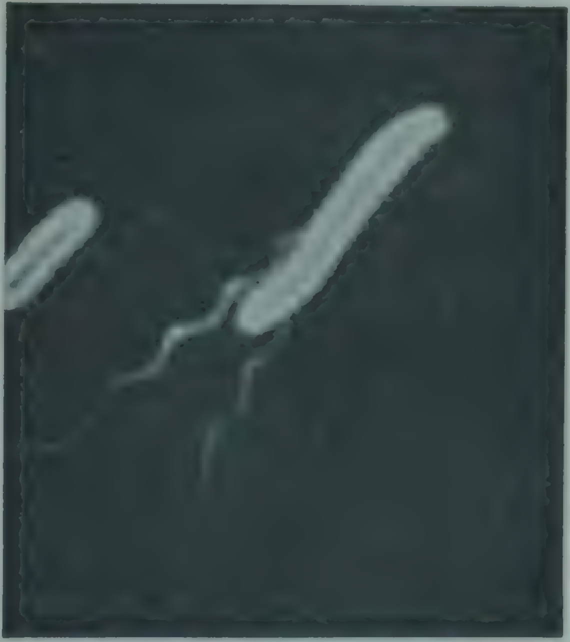

Fig. 50. Bacterium with split tail. Note spiral shape. × 5,000. (Pijper, Adrianus, J. Bact., vol. 53.)

Bacterial flagella easily become detached and are so fine as to be visible only when stained. The staining of these organs is rather difficult and this may be regarded as evidence that they differ in some way from the body or *somatic* part of the organism, the latter being easily stained.[10] This point will be referred to again later. It is of interest to note, in this connection, and with reference to Pijper's views, that stains for flagella also stain capsules well. A useful means of staining flagella is that recommended by Gray.* It must be pointed out that students will ordinarily not find it as easy to stain flagella as these directions make it appear. The factors involved are not well understood. However, a little perseverance is usually rewarded with success. To accomplish the best results the slides must be clean and free from grease and scratches, and the cultures must be carefully prepared. Some suggestions are as follows:

(*a*) Place slides in cleaning solution overnight; wash; dry from alcohol, *or*
(*b*) Cover with moistened Bon-Ami; dry, wipe with clean cloth.
(*c*) *Moist* agar slant cultures 12 to 24 hours old are best, although broth cultures often show flagella well. In case one fails, try the other.
(*d*) Make light emulsion in sterile distilled water and incubate 10 to 15 minutes to develop and extend the flagella fully.
(*e*) Place large loopful on slide and allow to spread by tilting.
(*f*) Do not *fix* by heat.

Capsule, Slime Layer, or Sheath. Outside of the cell wall proper there appears to be on most bacteria a layer of slimy, viscous or gelatinous substance varying in thickness, consistency and chemical composition according to species, growth conditions, phase variation or genetic characters which will be discussed later on. The electron micro-

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* *Gray's method:*
I. Mordant:

Solution A

Potassium alum (sat. aq. sol.)	5.0 ml.
Tannic acid (20 per cent aq. sol.) (This must be preserved in the ice box with a few drops of chloroform if a large amount is kept on hand.)	2.0 ml.
$HgCl_2$ (sat. aq. sol.)	2.0 ml.

Solution B

Basic fuchsin (sat. alc. sol.)	0.4 ml.

Mix solutions A and B only on the day they are to be used. Separately they keep well, but deteriorate after mixing.

II. Stain:
Carbolfuchsin as prepared for Ziehl-Neelsen stain.
1. Flood smear with mordant and allow to act for 5 minutes. Rinse off gently.
2. Flood with carbolfuchsin for 5 minutes. Do not heat. Rinse off gently.
3. Blot dry.

scope has aided greatly in the study of the slime layer or capsule, especially in those species in which it is a very thin sheath.*

The relation of capsule to cell is not known definitely. In some cases capsules seem to be a thickened layer of the cell wall; in others, a secreted mucoid substance not part of the cell but adhering to it, the amount adhering depending on solubility in the surrounding medium. Some bacteria secrete much slimy substance into their environment. This may not be a true capsule and may bear no more true structural relation to the cell proper than any excreted waste product. A collection of organisms embedded in their secreted slimy substance is often referred to (improperly) as a zooglea or zoogleal mass. The difference between zoogleal material and true capsules is not entirely clear.

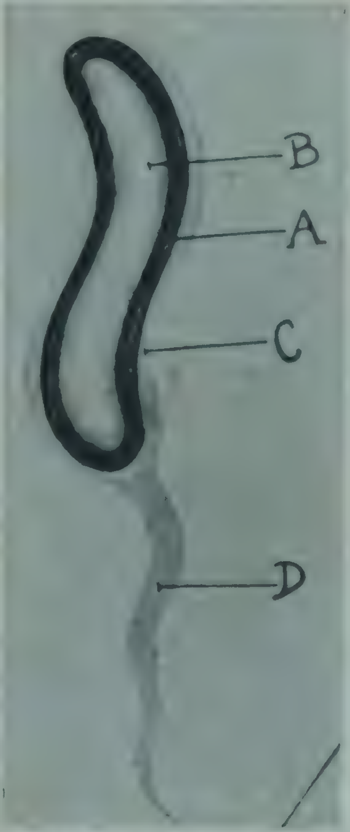

Fig. 51. Diagram of typhoid bacillus, showing cytoplasm (B), cell wall (A), slime layer (C) and wavy tail (D). (Pijper, Adrianus, in J. Path. and Bact., vol. 58.)

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* Some organisms on which a capsule is not discernible may nevertheless possess the same chemical substance, as determined by immunological tests. It appears to be closely linked with the cell and may be inapparent merely because it is not formed in great quantity. These surface carbohydrate complexes are of great importance as we shall see later on.

In most species large, easily seen capsules seem to be called forth only by certain particular circumstances of environment such as growth in the animal body, or on certain media. Some, for example, *Leuconostoc,* form their capsular material best in sugar vats or in milk. There is good reason to believe that capsule formation may be stimulated by unfavorable environments such as the resistance of an infected host. For example, capsules are often found well developed on, and demonstrably correlated with, virulence of pathogenic forms such as pneumococci and the organisms of whooping cough, gonorrhea, meningitis, anthrax and other diseases. The capsules seem to enable these bacteria to evade the defensive mechanisms of the infected body. The bacteria often cease forming capsules when removed from the body to laboratory test-tube media.[11]

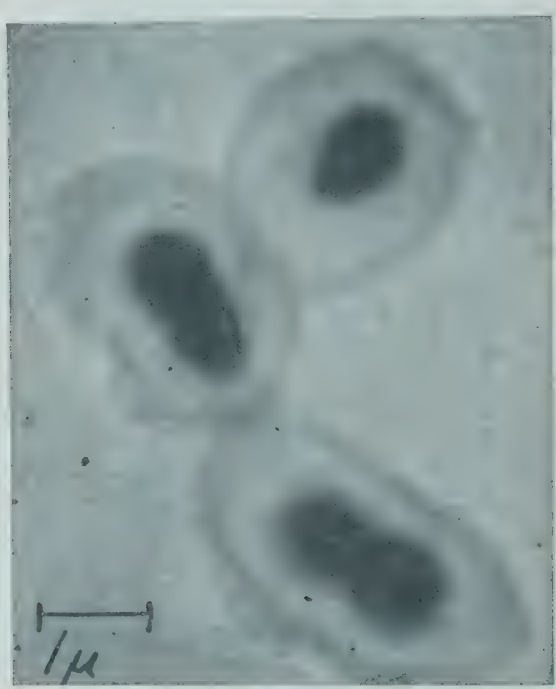

Fig. 52. Pneumococcus, showing capsules. (From Mudd, Heinmets and Anderson, J. Exper. Med., 1943.)

The chemical composition of capsules varies widely with species and they contain substances of great interest to the microbiologist. Most are composed mainly of carbohydrates or gums; others also contain nitrogen and phosphorus and may be of the nature of mucus or the mucins. Still others may be largely composed of inorganic gel-like substances. As will be emphasized in the discussion of streptococci and pneumococci, capsular substance has specific chemical properties which enable us to distinguish immunologically between various closely similar types of organisms which could not otherwise be differentiated.

DEMONSTRATION OF CAPSULES. There are several methods of demonstrating the presence of capsules on bacteria. As the capsules differ so greatly in composition, however, they are not all amenable to the same process.*

Capsules, like flagella, do not have a marked affinity for basic dyes and some are easily removed by mechanical means or by washing with water. Hence great care is necessary in demonstrating them by staining methods.

Cell Wall. At this point a definition of terms is desirable, as pointed out by Knaysi. By cell wall is meant the thin, sharply defined, relatively tough or inelastic (though flexible) structure inside the slime layer or capsule, physically and chemically distinct from that layer and from the true cytoplasm of the cell inside. The various structures are beautifully shown by special staining procedures and by the electron microscope.

The cell wall varies in thickness, strength and chemical composition with species and growth conditions and other unknown factors. It may be thought of as being like thin paper in consistency; or better, like cellophane, since it is transparent. On some cells it is not readily demonstrated; in others (especially organisms of the genus *Bacillus*) it is easily seen under proper conditions of observation. The electron microscope is most useful in this connection.[12]

The chemistry of the cell wall is not well established. In general it appears to be rather stable and resistant to the action of most substances except strong acids, alkalis and the like. Most studies indicate the presence of carbohydrates, *e.g.*, cellulose, or carbohydrate complexes such as chitin-like compounds, mucinoids, hemicellulose and similar substances.

The roll of the cell wall, acting in conjunction with the other sur-

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* *Capsule stain* (*Welch method* [modified]):

A. Crystal violet	
Saturated alcoholic crystal violet	5 ml.
Distilled water	95 ml.
B. 10 per cent $CuSO_4$	1 liter

1. Make thin smear.
2. Dry in air.
3. Fix by gentle heat.
4. Cover with crystal violet solution.
5. Heat to steaming for 1 second. (Do not boil!)
6. Use no water.
7. Rinse *immediately* and *copiously* with copper sulfate solution.
8. Use no water.
9. *Blot* dry *immediately*.
10. Examine.

The organisms are stained purple and the capsule appears as a faintly blue halo.

face structures (capsule and cytoplasmic membrane) is of critical importance.

Methods* of differentiating the cell wall and cytoplasmic membrane have been described by Knaysi[13] and by Dyar.[14]

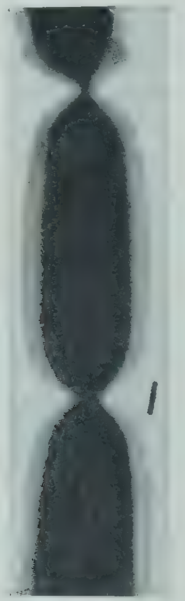

Fig. 53. Twenty-four hour culture; cells treated with 0.5 per cent boric acid 1 hour. Cell wall is clearly shown surrounding what appears to be healthy protoplasm. × 10,000. (Evans, Charles A., and Underdahl, Norman R., in J. of Bact., vol. 53.)

Whatever the composition of the cell wall it is, in all the bacteria, one of the membranes through which must pass not only the nutrient material for the cell, but also all waste matter, enzymes and other

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* *Stain for cell membrane* (*Dyar*).

1. Smear, dry and fix by gentle heating.
2. Add 3 drops of 0.34% aqueous cetyl pyridinium chloride.
3. To this, mixing on the slide, add 1 drop saturated aqueous Congo red.
4. Wash gently with water.
5. Counter stain with methylene blue for a few seconds. Wash.

The cell wall is stained a light red, the cytoplasm blue.

Stain for cell wall, etc. (Knaysi).

Prepare smear from young culture.
Fix by warming.
Apply mordant† 10 minutes.
Stain with a drop of carbol fuchsin‡ under a coverslip.

† Mordant

Potassium alum. saturated aqueous	70 ml.
Tannic acid, 20%	30 ml.

This should be renewed each 2 weeks.

‡ Ziehl-Neelsen's acid-fast stain (see page 45).

products given off by the cell during its life. The membrane is *permeable* to these substances, but impermeable to others and is therefore said to be a *semipermeable membrane*. In order to retain within the cell the proper substances in the proper proportions, and to prevent the entrance and allow the escape of improper or waste substances, it must exert a very fine discrimination or selective permeability at all times. In this it is doubtlessly aided by the cytoplasmic membrane.

Any slight defect or alteration in the discriminatory properties of the cell wall must, therefore, have a profound effect on the cell contents. When, as is the case with bacteria, the entire individual consists of the cell contents and depends for its existence as a living entity on the chemical and physical equilibrium of its protoplasm, then the maintenance of the integrity and uniformity of the cell wall is a matter of the greatest moment. Substances or forces which tend to alter the wall or overcome its selective powers must influence the well-being of the cell. These will be discussed later on.

Cytoplasmic Membrane. Since in plasmolysis the cytoplasm shrinks from the cell wall, it appears to be enclosed in an exceedingly thin membrane which is normally in contact with the inside of the cell wall. The chemical nature of this membrane is not clear but it may consist of a condensation or slight thickening of the cytoplasm itself where it is in contact with the cell wall. The remarks on the importance of selective semi-permeability and alterations in structure made in connection with the cell wall also seem to apply to the cytoplasmic membrane. The two are undoubtedly of fundamental import in acting as mediators between the living cytoplasm within, and the world outside.

Cytoplasm. This is one part of the actual, living material of all cells. The other principal part, in cells of plants and animals more complex than bacteria, is the nucleus. The cytoplasm is usually clear, watery or slightly viscous, and homogeneous in appearance although granules of various substances, such as stored food, oil, etc., may be suspended in it. It consists of a complex mixture including proteins, lipoids, carbohydrates, minerals and water. The organic matter is largely in the colloidal state, and so intimately intermingled as to defy synthesis and any analysis but the grossest sort. The energy released by the chemical and physical reactions occurring among the colloidal and other materials within the cytoplasm and nucleus and at their surfaces is manifested as LIFE.

THE CYTOPLASM: COLLOIDS. A substance is in a *colloidal state* (sometimes called the fourth state of matter) when it occurs in such finely divided particles (the disperse or discontinuous phase) that they remain suspended in gas, water or other fluid (the continuous phase) indefinitely. Such suspensions in fluids may be clear or "smoky" or milky in appearance but have, in general, a superficial resemblance to true solutions. An example of a colloidal suspension in a gas is smoke. Smoke is composed of minute particles suspended in air. Milk is a colloidal suspension of casein and butter-fat in whey. Many substances, including various metals, can be made to assume the colloidal state. Colloidal particles vary in size but are probably made up of small groups of molecules or atoms. Their small size is important because when a given weight of a substance is subdivided into very tiny particles its surface is greatly increased and, as we shall see, *many biological phenomena depend on chemical and physical reactions occurring on surfaces.*

SURFACES OF COLLOIDS. A cube measuring 1 cm. along each edge presents a surface of 6 sq. cm. Cut in half, 8 sq. cm. are presented. In 10 slices, 24 sq. cm. are obtained, while if cut into particles almost as small as molecules, the surface is relatively enormous. Thus, a very small volume of matter may, by being extremely finely divided, acquire a very large surface. This is the state of affairs with colloids. This surface development is very important, since forces active at surfaces greatly affect colloids.

CYTOPLASM AND PROTOPLASM A COLLOID. Protoplasm (roughly all the living cell substance enclosed in the cell membrane) is principally proteinaceous matter in a colloidal state, the minute proteinaceous particles being suspended in an aqueous matrix containing, in solution, various salts, organic substances, nutritive compounds which have diffused inward from the surrounding medium through the cell membrane, and waste substances, which will diffuse outward and be liberated into the medium. Between the colloidal particles and the suspending fluid inside the cell there is a constant interplay and exchange of various atoms, molecules, electrical forces, physical stresses, chemical attractions, and so on, all in a slightly unbalanced state of equilibrium with respect to each other, the net resultant of all being life, reproduction, motility, fermentation, putrefaction, luminescence, toxin production, and all the phenomena familiar to the microbiologist. Obviously, any abnormal alteration, however slight, in the physical or chemical constitution of the contents of the cell will profoundly affect the functioning of the cell and may result in its death. It is necessary for the microbiologist to be informed as to the agents which bring about such changes, both that he may avoid them in attempting to cultivate bacteria, and that he may make use of them intelligently in disinfection and sterilization.

Nucleus. At this point we stumble upon an interesting controversy. Neither the nature nor the existence of the nucleus of bacteria is certain. Because the matter is still unsettled we will here mention some of the difficulties in the way of arriving at a satisfactory solution and give only the principal theories concerning it.

The chief difficulties are, first, the minute size of bacteria. A nucleus, if it existed, would be barely within the limits of visibility of the best of light microscopes. This difficulty has been in part overcome by the use of the electron microscope. Second, assuming a nucleus to be present and large enough to be seen, it is difficult to distinguish between it and various sorts of food granules, such as volutin, or desoxyribonucleic acid. Methods of staining nuclei in cells of large plants and animals, protozoa, yeasts and molds, when applied to bacteria, yield data which while very suggestive, fail to give conclusive evidence.

It is not difficult to stain and see various internal structures. The difficulty lies in proving their nuclear nature. Studies of dividing cells show no true mitotic figures. Even though masses of material staining like nuclei may localize in two different parts of a cell in equal amounts and symmetrically arranged, and accompany those parts when the cell divides, this is no proof of their identity as nuclei, since masses of inert chromatic material such as volutin also undergo such divisions. Studies of heredity in bacteria have failed to demonstrate that mendelian laws hold true. This, as well as failure to demonstrate sexuality, would seem to be a fundamental difficulty in the way of demonstrating nuclei in bacteria. Also, the difficulty must be borne in mind that the structures responsible for nuclear functions may very well differ in different species, being more like true nuclei in some, less so in others. This may account in part for the differences of opinion in this field.

The principal views on bacterial nuclei (except for those denying the presence of any nucleus) may be summarized as follows:

1. *There is a true nucleus and cytoplasm.* This has been discussed above.

2. *There is a true nucleus but no cytoplasm.* Reasons have already been given for doubting the existence of a true nucleus of the sort clearly differentiated in higher cells. The idea that there may be a nucleus but no cytoplasm arises from the necessity of explaining nuclear *functions* (life, growth, fission, maintenance of considerable stability of heritable characters, etc.), coupled with the facts that the bacterial cell *as a whole* stains like nuclear material and that no differentiation between this material and cytoplasm can be made. These difficulties may be reconciled by some form of the following idea:

3. The nucleus consists of minute particles of nuclear material, or granules of chromatin, diffusely scattered in the cytoplasm, perhaps attached to a mitochondrial reticular net work of threads; or perhaps strung on a long mitochondrial thread, the whole resembling a tangled string of beads enclosed within the cytoplamic membrane. In order to explain the appearance in electronographs of fairly large, discrete bodies said to be nuclei or analogous structures, it has sometimes been suggested that the scattered nuclear granules may coalesce into functional nuclei just before cell fission. This is a not unreasonable view and is consistent with many observations.[14a, 15]

By the use of special staining methods dark, symmetrical bodies can often be seen in bacteria. Their nuclear nature is largely an assumption.

The Feulgen reaction[*, 16] is cited by some microbiologists as a specific reagent for nuclear matter (thymonucleic acid) but is thought by others to be not specific. It depends on the fact that, on hydrolysis of thymonucleic acid, aldehyde groups of the desoxyribose portion of the nucleic acid are left free. These impart a violet color to the reduced (decolorized) fuchsin in Schiff's reagent. Non-nuclear com-

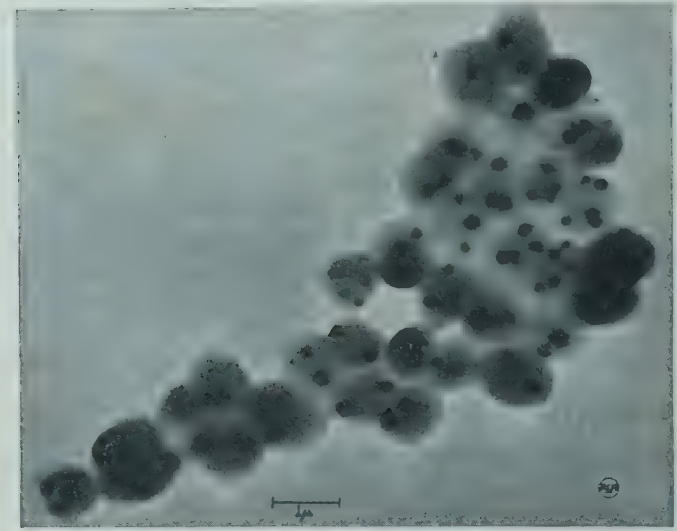

Fig. 54. Electronograph of micrococci (staphylococci) showing dark internal structures which may be nuclei. (Dr. George Knaysi, Cornell Univ., and Dr. Stuart Mudd, Univ. of Pennsylvania, in J. Bact., 1945.)

[*] *The Feulgen reaction*
Fix in 95% C_2H_5OH for 48 hours.
Apply chloroform for 30 seconds to the moist smear.
Dry in air.
Immerse in N/1 HCl, for 10 to 15 minutes at 60° C.
Pass through cool N/l HCl.
Soak in Schiff reagent[†] in the dark in a closed vessel 2 to 6 hours.
Wash gently in 1% bisulfite solution, then in water.
Dry and counterstain with a weak, green, dye solution.
Examine under oil (or mount under a coverslip with 1% bisulfite solution and examine in the wet state).

[†] *Schiff reagent*
Fuchsin (basic) 1 gm.
H_2O 200 ml.
} shake, warm, filter
To the filtrate add:
N/l HCl 20 ml.
Anhydrous Na bisulfite 2 gm.
Stopper tightly and stand 24 hours in dark place.
For a more sensitive, colorless Schiff reagent see Tobie, W. C.[19]

pounds of nucleic acid in the cytoplasm are said to give a different, more reddish color.[17] Various non-nuclear aldehydes, aldohexoses and aldopentoses give an intensely positive reaction, also the cell wall and other parts of the cell.[18] Thus the reaction is not wholly specific.

Thymonucleic acid, which seems to be characteristic of nuclei, has been found in preparations of supposed nuclei removed from staphylococci. This is Feulgen positive. So also is desoxyribonucleic acid.

Spores. Only two genera of true bacteria (*Bacillus* and *Clostridium*) form spores. Some yeasts also form spores. Sporelike bodies called *conidia* are produced by molds and certain species of the order *Actinomycetales* (see p. 461). Conidia of yeasts, molds and Actinomycetales are not very heat-resistant and are produced as a means of reproduction.

When a rod of the genus *Bacillus* is to sporulate, a tiny refractile granule appears in the cell. The granule enlarges and becomes glassy and transparent.[18a]

Just what goes into the granule is not clear. Some data indicate that it grows from a single primordium of nuclear material; others that it represents an aggregation of nuclear granules. It has been suggested that spore formation is accompanied by sexual functions, such as automixis, or nuclear fission or fusion. Whether this is so or not, part or all of the cell content is finally condensed and surrounded by a thick shell, which may represent a much thickened cytoplasmic membrane.

The state of the water in the spore is not settled. There is evidence that the spore content is dehydrated. Newer data indicate that the water content is unchanged but that the water in the spore is not in a free state, being bound or imbibed by the lyophilic colloids in the spore.[20] This, as well as an increased calcium content of spores[21] could account in part for the heat resistance of endospores. The apparent dryness of a hard-boiled egg is a good illustration of bound water.

The spore wall, as well as the cell wall, are shown in electronographs made by Evans and Underdahl[22] and by Knaysi, Baker and Hillier.[22a]

The empty cell membrane of the bacillus may separate off, like the hull of a seed, leaving the spore as a free, round, or oval body. The empty membrane is called a *sporangium*. Disinfectants cannot readily penetrate into spores, nor can aniline dyes. A rod with a spore inside, when stained by ordinary methods, may thus appear to have a hole in it (Fig. 55). However, the outer shell of the spore may readily be colored so that in stained smears free spores may appear as tiny, blue rings or ovals.

Function of Spores. Sporulation of bacteria obviously is not a reproductive function since most species reproduce perfectly without demonstrable sporulation. Further, in bacteria, as a rule, only one spore is produced by each cell and this spore produces no *new* individual. In this respect bacterial spores differ from the spores of true fungi and higher plants.

There is little or no metabolic activity in a spore, since all metabolism depends on a suitable state of hydration. Not until moisture is present do bacterial spores germinate. They represent merely a dormant and resistant state which enables the bacteria producing them to survive during periods unfavorable to active vegetation.

When dry spores are placed in a suitable nutrient solution, the protoplasm within the shell imbibes water, swells, bursts forth and goes on as before. This process is beautifully illustrated by Knaysi, et al.[18a] (Fig. 56). Germination is greatly interfered with by the presence of some fatty acids.[22a]

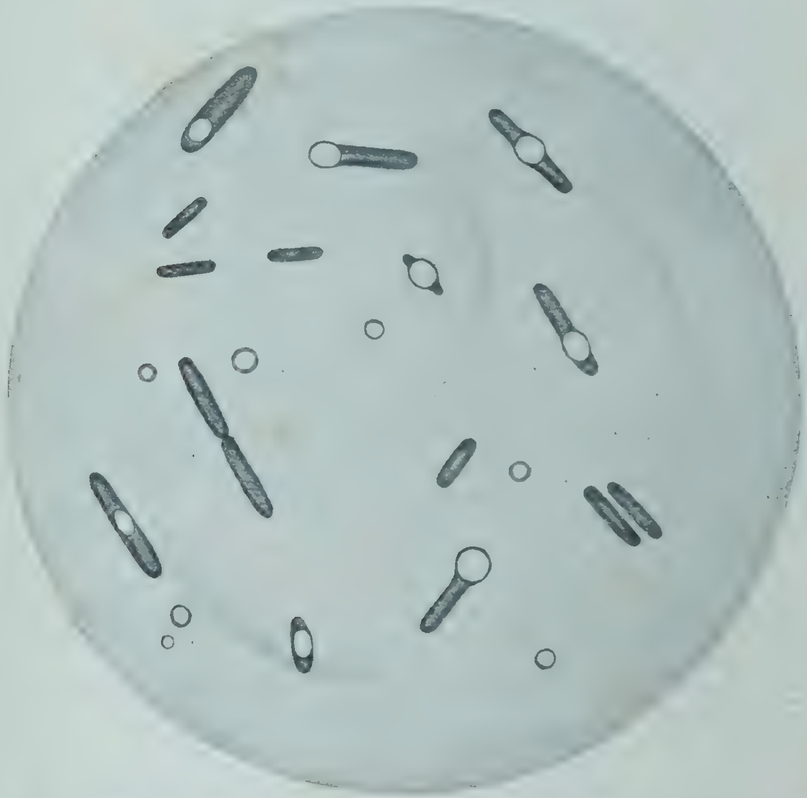

Fig. 55. Various types of bacterial spores. Some of the spores have escaped from the sporangia, stained with methylene blue, which does not penetrate inside the spore. Only the outer surface of the spores is stained.

Cause of Sporulation. Just what induces a given cell to go into the dormant spore stage is unknown. Sporulation is not necessarily a response to unfavorable conditions, since spores are often formed early in the life of a culture while conditions are wholly favorable to continue vegetative activity. It may be that sporulation occurs at a certain age of the individual cell and represents a sort of resting and rejuvenating process, but this has not been shown to be the case.

Resistance of Spores. When protein or protoplasm is moist or well hydrated, as is its condition in an actively growing, "vegetative," or live condition, it coagulates readily and is therefore vulnerable with respect to heat, chemicals, etc. The "white" of egg is a good illustration of a hydrated protein. It coagulates ("hard boils") readily enough.

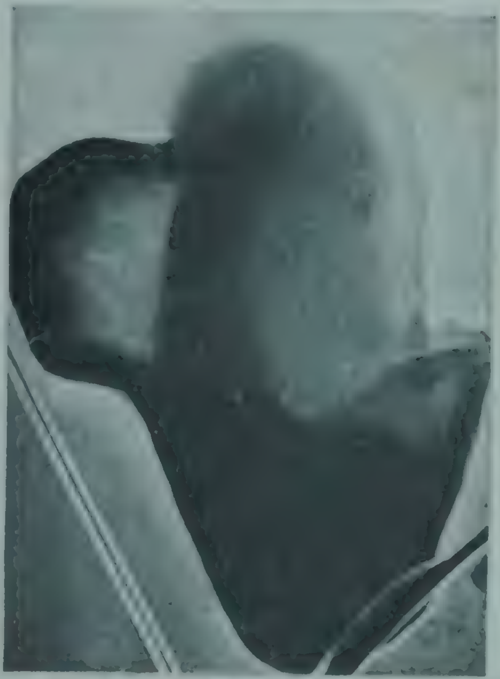

Fig. 56. Electronograph of B. mycoides showing rupture and germination of a spore. (Courtesy of Knaysi, Baker and Hillier, J. Bact., 53.)

When protein is dehydrated, or the protein and water are bound, it is no longer readily coagulable. It is attacked only with difficulty by various chemicals, and withstands high temperatures and other conditions which would soon destroy it were it allowed to become hydrated. Egg powder is a good example.

In this way spores can survive where the active, vegetative, or hydrated stage would soon perish. So resistant are some bacterial spores that they will live for as long as twenty years or more on dry splinters of wood, grow after treatment in strong disinfectant solutions, survive an hour or more of boiling, and remain unharmed by an hour or more in a hot oven.

Staining Spores. Although ordinarily resisting the penetration of dyes, spores may, nevertheless, be stained if proper methods are employed. A number of spore stains are available; the very simple method of Schaeffer and Fulton is extensively used.*

It is sometimes difficult or impossible to demonstrate spores by means of the microscope, even when staining methods are employed. Resort must then be had to tests for resistance of the culture to heat. A culture which resists 85° C. for 20 minutes almost certainly contains spores.

Granules. In many bacteria stained by ordinary methods, granules are readily seen (Fig. 57). They are distinguished by the fact that they stain more intensely than the rest of the cell, or are of a different color. They are often spoken of as *metachromatic* granules. We have already mentioned a number of kinds of granules.

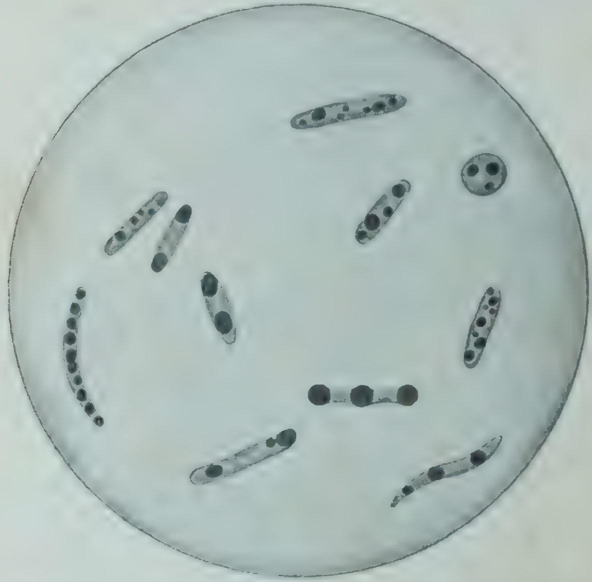

Fig. 57. Various species of bacteria showing granules of volutin, fat, starch, etc., in characteristic arrangements.

The composition and function of these granules is, for the greater part, a matter of debate (Fig. 58). As shown by their reaction to various test reagents, such as iodine, some of them are of starch (blue), glycogen (brown) or closely related compounds and doubtless often represents reserve food substances since they tend to disappear during periods of food scarcity. In some species pure elemental sulfur serves the same purpose. This may be demonstrated by smearing the bacteria on a slide, and treating with carbon disulfide. After evaporation of the disulfide the sulfur remains in characteristic crystal forms. In many bacteria, granules of a nitrogenous compound, *volutin*, are commonly observed. This has often been confused with nuclei. Volutin is a food

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* *Schaeffer and Fulton method* (modified):
A. Five per cent aqueous malachite green.
B. Five per cent aqueous eosin.
1. Flood smear with malachite green solution.
2. Heat over steam bath for 5 minutes (do not boil).
3. Wash gently with water.
4. Flood smear with eosin solution.
5. Rinse and blot dry.

Spores green, bacillary bodies red.

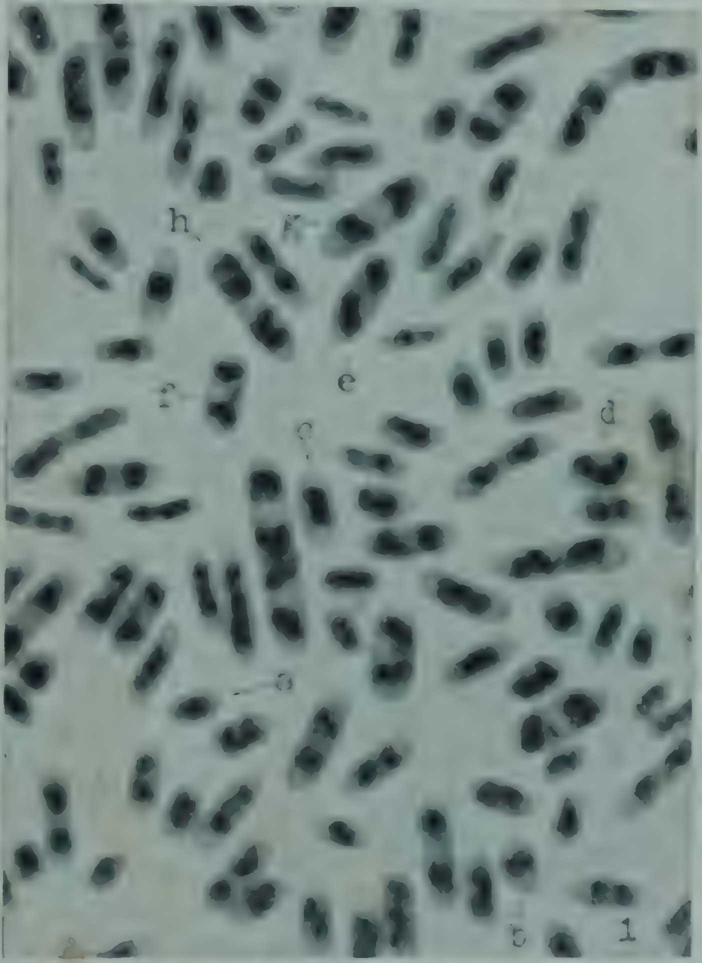

Fig. 58. *a–h* are successive stages in the development of a coccoid cell with a broad (double?) central chromatinic structure into a typical rod-shaped two-cell bacterium with two pairs of chromosomes. Note the V-shaped division stage at *d*. The chromosome forming the left limb of the V is so much broader than the right one that it seems plausible to assume that it is preparing to divide. At *e* a thin strand of chromatinic matter is seen connecting recently divided chromosomes. (Robinow, C. F. in Cambridge J. Hygiene, vol. 43.)

material related to, or consisting of, nucleic acids. These are not identical with the protein of the nucleus, as they are dissolved in hot water (80° C.) instead of coagulated by it, and are not digested by trypsin.

Staining Granules. There are many methods of bringing granules into prominence, the method depending largely on the chemical nature of the granules themselves. One commonly used to demonstrate volutin granules is that of Albert.*

Fat Granules. Some bacteria synthesize and store much fat, especially if fed with sugar or other fermentable food—in this showing a striking resemblance to many human beings.[23] Some species of the genus *Bacillus* are outstanding in this respect, but other bacteria, yeasts and fungi also store up much fat (see Fig. 59). A 24-hour culture on glycerol agar, if properly stained, as by Burdon's method†, [24] will show large amounts of fat. The method has been relatively little studied and offers a good field for investigation of problems in metabolism, morphology, taxonomy, etc.

Chemical Composition of Bacteria. In spite of their supposedly primitive nature, bacteria are not markedly different in chemical composition from other living cells. However, the exact chemical analysis of *living* matter is difficult, if not impossible, since to analyze it one must kill it and disturb the interrelations of various components of

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* *Albert's method of staining volutin granules:*

A.	Toluidin blue	0.15 gm.
	Methyl green	0.20 gm.
	Acetic acid (glacial)	1.00 ml.
	Alcohol (95 per cent)	2.00 ml.
	Water	100.00 ml.

Allow to stand 24 hours. Filter through paper.

B. Iodine as in Gram's stain.

1. Prepare smear as usual.
2. Flood with solution A for 1 minute. Wash with water and blot dry.
3. Stain with solution B for 1 minute, wash and blot dry.

Granules black; other parts dark or light green.

† *Fat stain* (Burdon)

1. Prepare the film, dry and fix by gentle heating as for the Gram stain.
2. Flood the entire slide with Sudan black (0.3 per cent dry dye in 70 per cent C_2H_5OH) and leave the slide for 5 to 15 minutes. Drying of the dye will not harm the preparation. Use of a Coplin jar is less satisfactory.
3. Drain off excess dye and blot dry.
4. Clear in xylol for about 1 minute, dipping in and out.
5. Counterstain with 0.1 per cent aqueous safranin for 10 seconds. Overstaining with counterstain will mask the fat stain. It may even be omitted.

The fat appears as dark, blue-black masses; the rest of the cell is pink.

The stock of solution of Sudan Black keeps well in a clean, stoppered bottle. It should be occasionally shaken and allowed to settle overnight before use. Sudan III and Scharlach R are *much less* satisfactory.

the cell. The composition of bacteria appears to be much like that of other plant cells.

Bacterial protoplasm is composed chiefly of the elements C, O, H, S, N and P, with smaller amounts of many other elements as Na, K, Ca, Fe, Mg, Cl and so on. Species differ in the actual amounts of each element present. It is known that in the cell the ratio of the last group of elements and some of the first may vary with the composition of the medium on which the bacteria are growing at the time they are analyzed. This is true in a marked degree of various constituents of the bacterial cell.

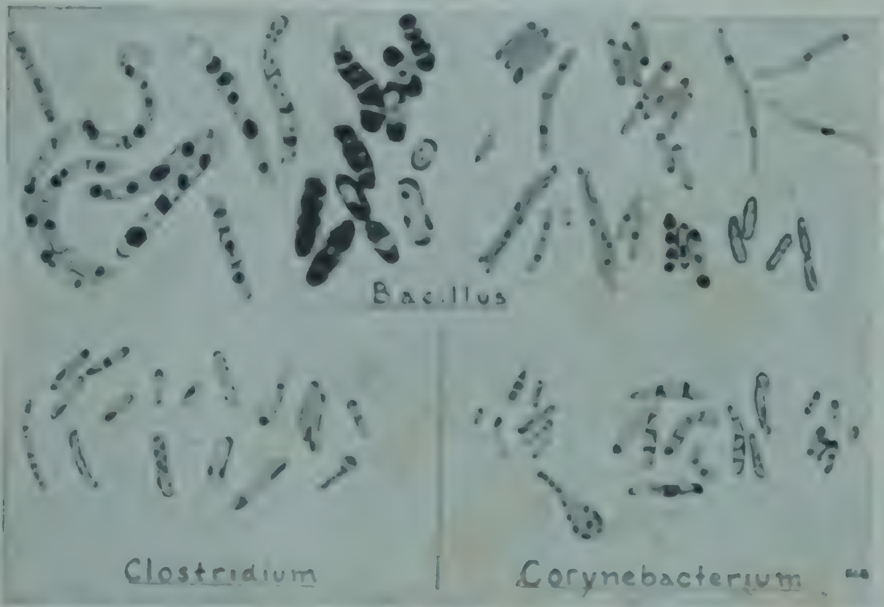


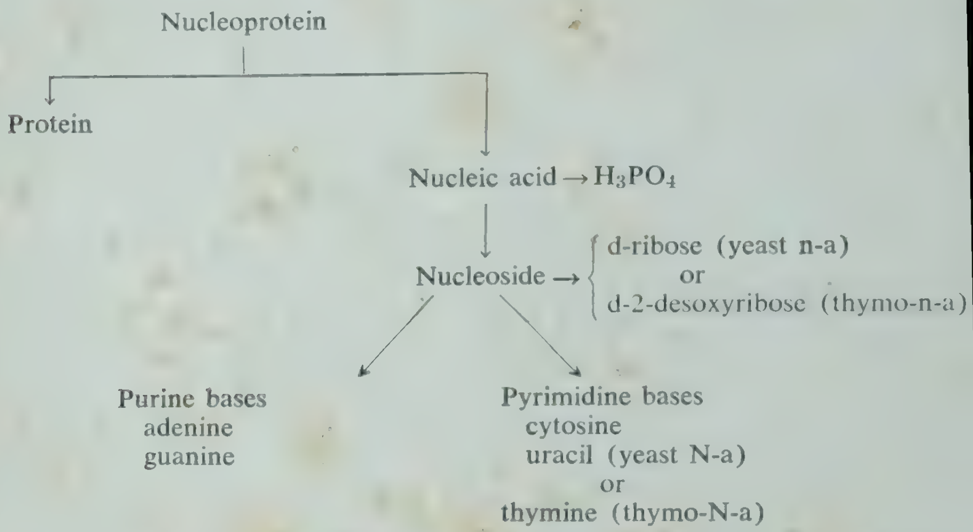
Fig. 59. Various species of bacteria stained for fat with Sudan black. Note the similarity of many of these masses to pictures representing what is said to be nuclear material. (Burdon, K. L., in J. of Bact., vol. 52.)

In bacterial protoplasm, as in other protoplasm, water is an important component, constituting roughly from 75 to 98 per cent of the whole weight and quite variable, depending on environment. The ash content may vary in amount from 2 to 30 gm. per cent of the dry weight of bacteria; and protein substance, including globulins, nucleoproteins, etc., from 12 to 90 per cent of the dry weight. Many complex, non-protein, nitrogenous substances are also found, among them being various purine bodies, polypeptides, amines and amino acids.

Nucleoproteins are found in all plant and animal cells as well as in some viruses. As their name implies, they are proteins associated with the nucleus. They give a positive Feulgen reaction.

Nucleoproteins are of the greatest importance in the synthesis of enzymes and other complex structures. They are intimately involved in heredity mechanisms.

The general structure of nucleoproteins and nucleic acids may be seen in the following diagram which shows the products given off by hydrolysis:


Nucleoprotein
Protein
Nucleic acid → H3PO4
Nucleoside →
d-ribose (yeast n-a)
or
d-2-desoxyribose (thymo-n-a)
Purine bases
adenine
guanine
Pyrimidine bases
cytosine
uracil (yeast N-a)
or
thymine (thymo-N-a)

Amino Acids. Data on ten amino acids in nine species of microorganisms show the same ten amino acids in all species, but in somewhat varying quantities in the different organisms. These amino acids constitute ". . . qualitatively and quantitatively a stable and characteristic property of the cell under fixed conditions of growth." "The quantities of individual amino acids in microorganisms may vary with the growth medium, aeration, and age of the cells."[25] The amino acids are the same as those found in cells of higher plants and animals.

The carbohydrate content is also very variable since, as has been pointed out, many bacteria store up granules of polysaccharides for later use. Figures such as 12 to 35 per cent of total dry weight are found in various species and various analyses. Such carbohydrates as glycogen, starch, gums, pentosans, galactans, etc., have been reported, as well as more complex polysaccharides in the capsules of some species. The last are of great importance and are discussed more in detail later on in connection with certain specific problems. In only one species (*Acetobacter xylinum*) has true cellulose been demonstrated. In the "sulfur bacteria" sulfur (stored in elemental form) is an important constituent of the cells.

Lipids. In some genera, as that (*Mycobacterium*) containing acid-fast organisms like the tuberculosis bacillus, waxes are an important component. Fats and other lipids are present in nearly all cells to some degree in greatly varying quantities.[24]

Pigments of Microorganisms. Pigments are a prominent feature in many species of microorganisms. In most, their exact function is not known, nor is their chemistry, except in a general way. Chemically, the pigments may be grouped as:

a) Pyrrole compounds (Prodigiosin)
b) Carotenoid—lipids of red, yellow and orange shades (pigments of micrococci, etc.)
c) Melanins—soluble in strong acids and alkalis; (black and brown)
d) Anthocyanins and xanthines—water and alcohol-soluble pigments of red, blue and violet tints. Also greens and yellow colors
e) Phenazine derivatives (pyocyanin, etc.)
f) Cytochrome (a respiratory pigment)
g) Bacteriochlorophyll

The anthocyanins may be excreted as colorless compounds called *leucobases*, becoming colored only on oxidation after excretion. A good example is the blue pyocyanin (see page 573).

Pigments in general vary greatly in amount and intensity of color, even in the same organism. Growth at various temperatures and on various media affects either the mechanism responsible for pigment formation or the color of the pigment itself. Pigments develop best on solid media in close association with air. Anaerobic growth usually results in lack of, or discoloration of, bacterial pigment. Peptone or other nitrogen source seems to be essential to pigment formation. Pigmented species may give off nonpigmented variants.

Pigments may be intracellular, or they may pass through the semipermeable membrane of the cell and so be extracellular. *Prodigiosin*, the pigment of *Serratia marcescens*, is of the extracellular type (see page 575).

Function of Pigments. Except for the photosynthetic pigments, the physiological significance of pigments of microorganisms is not clear. There seems to be insufficient evidence to support the view that pigments protect microorganisms from light. That there is, however, a definite relationship between light and pigment formation is clear. Baker[26] showed that dead bacteria do not become pigmented on exposure to light, nor do alcoholic extracts of pigments. Many bacteria do not produce pigment in the dark but do so when growing in the light. Ultraviolet and sunlight irradiation cause some bacteria to produce pigment when subsequently growing in the dark.

With the possible exception of the blue pigment pyocyanin of *Pseudomonas aeruginosa*, and bacteriochlorophyll (see Chapter 25) pigments do not seem to play a part in respiratory process or in any synthetic process.

Bacteriochlorophyll. The most interesting and best known pigment of bacteria is the green bacteriochlorophyll of the autotrophic, alga-like "sulfur bacteria" (*Rhodobacteriineae*) and related species.[27] The pigment in these species is intracellular and has a function analogous to that of chlorophyll (see section on Rhodobacteriineae, page 450).

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Chapter 5

CLASSIFICATION OF THE SCHIZOMYCETES

When Leeuwenhoek discovered microscopic forms of life in the seventeenth century he grouped them all—protozoa, bacteria, yeasts, and possibly certain forms of algae—under one head, "animalcules." As knowledge of "animalcules" grew it became evident that they were not all alike. In order to discuss them intelligibly it became necessary to draw up descriptions of the different sorts. Names were given to certain well-defined organisms in order to simplify discussions. As information increased, it became evident that certain of the well-defined organisms were in general similar, while certain others could easily be differentiated from them. Then it became convenient to classify and name the various major groups, and to make lists of the types making up each. These were the first attempts at classification. The object of classification was, as it is now, to facilitate discussion and study by creating a set of uniform exact descriptions; to show the natural relationships of the creatures under investigation; and to provide a basis for description and classification of newly discovered types.

While all agree that classification is a desirable and necessary phase of biological work there is much disagreement as to the best *method* of classifying living things. The zoologists and the botanists have arranged matters in their fields fairly satisfactorily largely on the basis of morphological characters. These are a good guide to *natural* or taxonomic relationships because they are based on a long, connected history of evolutionary and phylogenetic development. The microbiologist, especially the bacteriologist, can only speculate as to the evolutionary and phylogenetic history of microorganisms; hence he has not arrived at taxonomic systems in which the *natural* relations of the organisms are used as a basis.

Nevertheless, he has systems of classification. They have been built up largely as arbitrary *descriptive keys for identification* of organisms according to whatever information about them may be available and cannot properly be called natural classifications. They are extremely useful, even indispensable, but their artificial and entirely arbitrary nature must not be forgotten.

A great deal of time, thought and labor has been expended by hundreds of workers in arriving at present-day systems of classification or descriptive keys, but there are still many difficulties.

Organisms may be arranged in order of importance, size, shape, or some other criterion. In dealing with small numbers having clearly defined characters on which to base the arrangement, this may not be too difficult a task. If, however, the concept of importance is used, a very grave difficulty arises. Whose opinion as to grades of importance

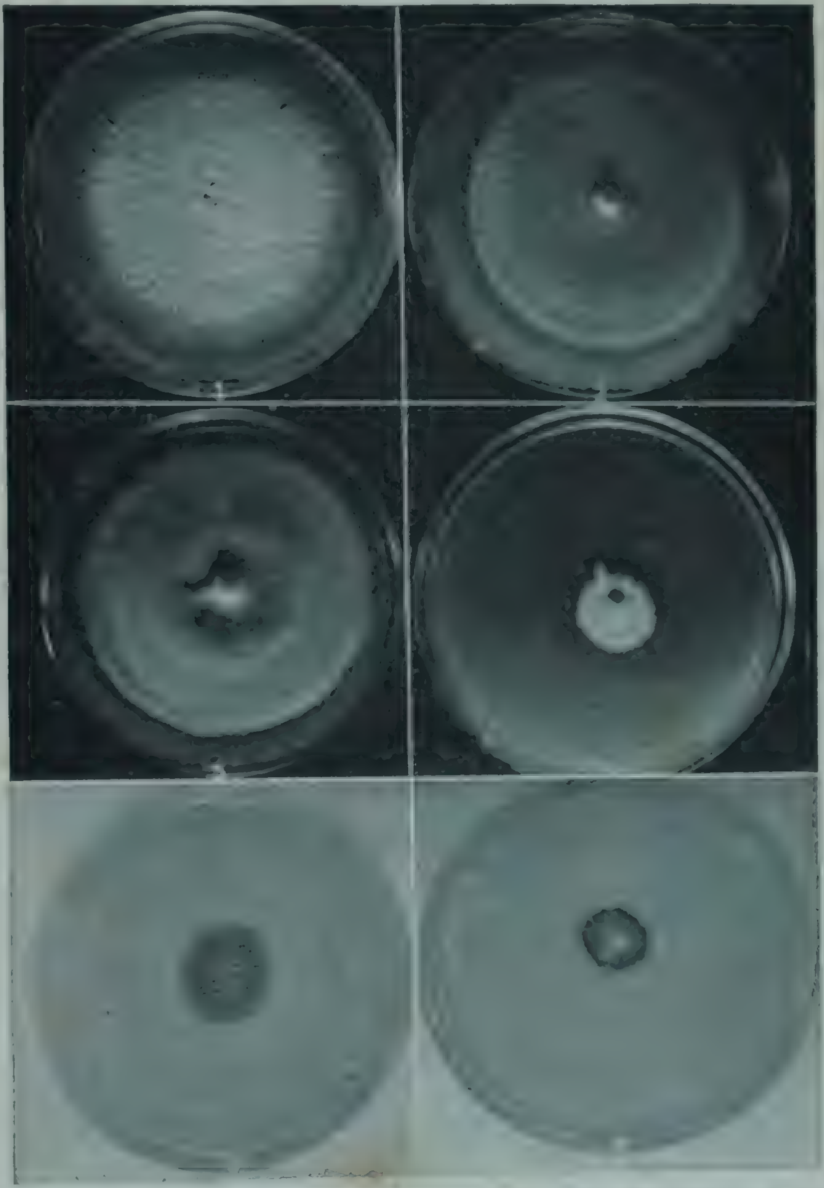

Fig. 60. Mutant growth forms of a fungus induced by irradiation. 1. Original strain of T. mentagrophytes. 2. Mutant A. 3. Mutant B. 4. Mutant C. 5. Mutant D. 6. T. violaceum. (Emmons, C. W., and Hollaender, A. in Arch. Derm. and Syph., vol. 52.)

is to be accepted? It is obvious that, if many organisms of nearly equal importance are involved, and more than one evaluation of degrees of importance are made, the orderly arrangement becomes a matter of personal opinion and may never be settled to the satisfaction of all. Added to this are the large numbers of other characters (biochemical, morphological, etc.), of all the different kinds of bacteria.

Even if certain characters were finally agreed upon, a new difficulty would arise because many of them are variable and an organism having one set of characters today may have others tomorrow. This would be a serious obstacle by itself, but it is still further complicated by the fact that some sorts of microorganisms may have some of the characters of others so that the taxonomist (one who attempts to bring order out of such seeming chaos) finally sets up arbitrary divisions among the creatures he is classifying and rules that the possession of certain characters shall differentiate between such and such organisms. This is convenient until it is found that certain of the creatures have lost some of those characters, but have acquired others. A further element of uncertainty is found in inaccurate or incomplete descriptions of some organisms so that they get put into the wrong group or seem to fit into no group. Finally, a man-made difficulty arises when it is found that by means of irrádiation with ultraviolet light it is possible to transmute one species of microorganism into another![1, 2, 3] (See Figure 60.)

Classification Schemes. Many systems of classification have been brought forward but none has remained long without revision and enlargement. One of the first schemes for bacteria was devised by Cohn in 1872 based on morphology only. In 1897 Migula devised a scheme based not only on form but color and some physiological characters like nitrogen fixation. Orla-Jensen in 1909 made up a system based largely on physiological properties and this has served as a model for all later schemes, of which that of Buchanan in 1925[4] is outstanding. A very excellent critique of the whole problem has been drawn up by van Niel.[5]

A system widely used by American bacteriologists and which has an international standing is published under the editorial direction of Breed, Murray, Hitchens, et al., in a volume generally known as "Bergey's Manual."[6] The book represents the collaborative effort of many of the world's best bacteriologists. This system, in 1948, divided the entire group of bacteria (Class Schizomycetes) into 5 major subdivisions or orders, which are outlined below. Each order is divided into families, and these into tribes, genera and species. Groups of similar species constitute genera; groups of similar genera constitute tribes, and so on. The names of orders end with the suffix *ales;* names of families with *aceae;* names of tribes end with *eae.*

Some names of species and genera are changed with successive revisions of the systems of classification, and different names are therefore sometimes used by different authors for the same organism, or two different organisms may be called by the same name. Thus there is some confusion in bacteriological literature. This is one of the signs of progress, like the confusion attendant on the repair of a downtown thoroughfare.

OUTLINE CLASSIFICATION OF THE SCHIZOMYCETES AND RELATED MICROORGANISMS*

- **Class SCHIZOMYCETES** Nägeli
  - ORDER I. EUBACTERIALES Buchanan
    - SUBORDER I. EUBACTERIINEAE Breed, Murray and Hitchens
      - Family I. *Nitrobacteriaceae* Buchanan
        - Tribe I. *Nitrobacterieae* Winslow et al.
          - Genus I. *Nitrosomonas* Winogradsky
          - Genus II. *Nitrosococcus* Winogradsky
          - Genus III. *Nitrosospira* Winogradsky
          - Genus IV. *Nitrosocystis* Winogradsky
          - Genus V. *Nitrosogloea* Winogradsky
          - Genus VI. *Nitrobacter* Winogradsky
          - Genus VII. *Nitrocystis* Winogradsky
        - Tribe II. *Hydrogenomonadeae* Pribram
          - Genus I. *Hydrogenomonas* Orla-Jensen
        - Tribe III. *Thiobacilleae* Bergey, Breed and Murray
          - Genus I. *Thiobacillus* Beijerinck
      - Family II. *Pseudomonadaceae* Winslow et al.
        - Tribe I. *Pseudomonadeae* Kluyver and Van Niel
          - Genus I. *Pseudomonas* Migula
          - Genus II. *Xanthomonas* Dowson
          - Genus III. *Methanomonas* Orla-Jensen
          - Genus IV. *Acetobacter* Beijerinck
          - Genus V. *Protaminobacter* den Dooren de Jong
          - Genus VI. *Mycoplana* Gray and Thornton
        - Tribe II. *Spirilleae* Kluyver and Van Niel
          - Genus I. *Vibrio* Müller
          - Genus II. *Desulfovibrio* Kluyver and Van Niel
          - Genus III. *Cellvibrio* Winogradsky
          - Genus IV. *Cellfalcicula* Winogradsky
          - Genus V. *Thiospira* Vislouch
          - Genus VI. *Spirillum* Ehrenberg
      - Family III. *Azotobacteriaceae* Bergey, Breed and Murray
          - Genus I. *Azotobacter* Beijerinck
          - Appendix: Genus A. *Azotomonas* Stapp
      - Family IV. *Rhizobiaceae* Conn
          - Genus I. *Rhizobium* Frank
          - Genus II. *Agrobacterium* Conn
          - Genus III. *Chromobacterium* Bergonzini
      - Family V. *Micrococcaceae* Pribram
          - Genus I. *Micrococcus* Cohn

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* From Bergey's Manual of Determinative Bacteriology. 6th Ed., 1948. The Williams and Wilkins Co., Baltimore, Md.

Appendix: Genus A. *Methanococcus* Kluyver and Van Niel
Genus B. *Pediococcus* Balcke
Genus II. *Gaffkya* Trevisan
Genus III. *Sarcina* Goodsir
Subgenera:
Zymosarcina Smit
Methanosarcina Kluyver and Van Niel
Sarcinococcus Breed
Sporosarcina Orla-Jensen
Family VI. *Neisseriaceae* Prévot
Genus I. *Neisseria* Trevisan
Genus II. *Veillonella* Prévot
Family VII. *Lactobacteriaceae* Orla-Jensen
Tribe I. *Streptococceae* Trevisan
Genus I. *Diplococcus* Weichselbaum
Genus II. *Streptococcus* Rosenbach
Genus III. *Leuconostoc* Van Tieghem
Tribe II. *Lactobacilleae* Winslow et al.
Genus I. *Lactobacillus* Beijerinck
Sub-genera:
Thermobacterium Orla-Jensen
Streptobacterium Orla-Jensen
Betabacterium Orla-Jensen
Appendix: Genus A. *Leptotrichia* Trevisan
Genus II. *Microbacterium* Orla-Jensen
Genus III. *Propionibacterium* Orla-Jensen
Genus IV. *Butyribacterium* Barker and Haas
Family VIII. *Corynebacteriaceae* Lehmann and Neumann
Genus I. *Corynebacterium* Lehmann and Neumann
Genus II. *Listeria* Pirie
Genus III. *Erysipelothrix* Rosenbach
Family IX. *Achromobacteriaceae*
Genus I. *Alkaligenes* Castellani and Chalmers
Genus II. *Achromobacter* Bergey et al.
Genus III. *Flavobacterium* Bergey et al.
Family X. *Enterobacteriaceae* Rahn
Tribe I. *Eschericheae* Bergey, Breed and Murray
Genus I. *Escherichia* Castellani and Chalmers
Genus II. *Aerobacter* Beijerinck
Genus III. *Klebsiella* Trevisan
Appendix: Genus A. *Paracolobactrum* Borman, Stuart & Wheeler
Tribe II. *Erwineae* Winslow et al.
Genus I. *Erwinia* Winslow et al.
Tribe III. *Serrateae* Bergey, Breed and Murray
Genus I. *Serratia* Bizio emend. Breed and Breed
Tribe IV. *Proteae* Castellani and Chalmers
Genus I. *Proteus* Hauser
Tribe V. *Salmonelleae* Bergey, Breed and Murray
Genus I. *Salmonella* Lignières
Genus II. *Shigella* Castellani and Chalmers
Family XI. *Parvobacteriaceae* Rahn
Tribe I. *Pasteurelleae* Castellani and Chalmers
Genus I. *Pasteurella* Trevisan

- Genus II. *Malleomyces* Pribram
- Genus III. *Actinobacillus* Brumpt
- Appendix: Genus A. *Donovania* Anderson et al.

Tribe II. *Brucelleae* Bergey, Breed and Murray

- Genus I. *Brucella* Meyer and Shaw

Tribe III. *Bacteroideae* Breed, Murray and Hitchens

- Genus I. *Bacteroides* Castellani and Chalmers
- Genus II. *Fusobacterium* Knorr
- Appendix: Genus A. *Fusiformis* Hoelling

Tribe IV. *Hemophileae* Winslow et al.

- Genus I. *Hemophilus* Winslow et al.
- Genus II. *Moraxella* Lwoff
- Genus III. *Noguchia* Olitsky, Syverton and Tyler
- Genus IV. *Dialister* Bergey et al.
- Appendix: Tribe *Mimeae* DeBord

Family XII. *Bacteriaceae* Cohn

- Genus I. *Bacterium* Ehrenberg
  - Subgenera:
    - *Kurthia* Trevisan
    - *Cellulomonas* Bergey et al.
    - *Saccharobacterium* Sickles and Shaw
    - *Agarbacterium* Angst
    - *Photobacterium* Beijerinck
    - *Methanobacterium* Kluyver and Van Niel
- Appendix: Overlooked Species and Synonyms

Family XIII. *Bacillaceae* Fischer

- Genus I. *Bacillus* Cohn
- Genus II. *Clostridium* Prazmowski

SUBORDER II. CAULOBACTERIINEAE Breed, Murray and Hitchens

Family I. *Nevskiaceae* Henrici and Johnson

- Genus I. *Nevskia* Famintzin

Family II. *Gallionellaceae* Henrici and Johnson

- Genus I. *Gallionella* Ehrenberg

Family III. *Caulobacteriaceae* Henrici and Johnson

- Genus I. *Caulobacter* Henrici and Johnson

Family IV. *Siderocapsaceae* Pribram

- Genus I. *Siderocapsa* Molisch
- Genus II. *Sideromonas* Cholodny

Appendix: Family *Pasteuriaceae* Laurent

- Genus I. *Pasteuria* Metchnikoff
- Genus II. *Blastocaulis* Henrici and Johnson

SUBORDER III. RHODOBACTERIINEAE Breed, Murray and Hitchens

Family I. *Thiorhodaceae* Molisch

- Genus I. *Thiosarcina* Winogradsky
- Genus II. *Thiopedia* Winogradsky
- Genus III. *Thiocapsa* Winogradsky
- Genus IV. *Thiodictyon* Winogradsky
- Genus V. *Thiothece* Winogradsky
- Genus VI. *Thiocystis* Winogradsky
- Genus VII. *Lamprocystis* Schroeter
- Genus VIII. *Amoebobacter* Winogradsky
- Genus IX. *Thiopolycoccus* Winogradsky
- Genus X. *Thiospirillum* Winogradsky
- Genus XI. *Rhabdomonas* Cohn

- Genus XII. *Rhodothece* Molisch
- Genus XIII. *Chromatium* Perty
- Family II. *Athiorhodaceae* Molisch
  - Genus I. *Rhodopseudomonas* Kluyver and Van Niel emend. Van Niel
  - Genus II. *Rhodospirillum* Molisch
- Family III. *Chlorobacteriaceae* Geitler and Pascher
  - Genus I. *Chlorobium* Madson
  - Genus II. *Pelodictyon* Lauterborn
  - Genus III. *Clathrochloris* Geitler
  - Genus IV. *Chlorobacterium* Lauterborn
  - Genus V. *Chlorochromatium* Lauterborn
  - Genus VI. *Cylindrogloea* Perfiliew

- ORDER II. ACTINOMYCETALES Buchanan
  - Family I. *Mycobacteriaceae* Chester
    - Genus I. *Mycobacterium* Lehmann and Neumann
  - Family II. *Actinomycetaceae* Buchanan
    - Genus I. *Nocardia* Trevisan
    - Genus II. *Actinomyces* Harz
  - Family III. *Streptomycetaceae* Waksman and Henrici
    - Genus I. *Streptomyces* Waksman and Henrici
    - Genus II. *Micromonospora* Ørskov
- ORDER III. CHLAMYDOBACTERIALES Buchanan
  - Family I. *Chlamydobacteriaceae* Migula
    - Genus I. *Sphaerotilus* Kützing
    - Genus II. *Clonothrix* Roze
    - Genus III. *Leptothrix* Kützing
  - Family II. *Crenothricaceae* Hansgirg
    - Genus I. *Crenothrix* Cohn
  - Family III. *Beggiatoaceae* Migula
    - Genus I. *Thiothrix* Winogradsky
    - Genus II. *Beggiatoa* Trevisan
    - Genus III. *Thiospirillopsis* Uphof
    - Genus IV. *Thioploca* Lauterborn
  - Appendix: Family *Achromatiaceae* Massart
    - Genus I. *Achromatium* Schewiakoff
    - Genus II. *Thiovulum* Hinze
    - Genus III. *Macromonas* Utermöhl and Koppe
- Appendix: Order *Caryophanales* Peshkoff
  - Family I. *Pontothricaceae* Peshkoff
    - Genus I. *Pontothrix* Nadson and Krassilnikow
  - Family II. *Arthromitaceae* Peshkoff
    - Genus I. *Arthromitus* Leidy
    - Genus II. *Coleomitus* Duboscq and Grassé
  - Family III. *Oscillospiraceae* Peshkoff
    - Genus I. *Oscillospira* Chatton and Perard
  - Family IV. *Caryophanaceae* Peshkoff
    - Genus I. *Caryophanon* Peshkoff
- ORDER IV. MYXOBACTERIALES Jahn
  - Family I. *Cytophagaceae* Stanier
    - Genus I. *Cytophaga* Winogradsky
  - Family II. *Archangiaceae* Jahn
    - Genus I. *Archangium* Jahn
    - Genus II. *Stelangium* Jahn

- Family III. *Sorangiaceae* Jahn
  - Genus I. *Sorangium* Jahn
- Family IV. *Polyangiaceae* Jahn
  - Genus I. *Polyangium* Jahn
  - Genus II. *Synangium* Jahn
  - Genus III. *Melittangium* Jahn
  - Genus IV. *Podangium* Jahn
  - Genus V. *Chondromyces* Berkeley and Curtis
- Family V. *Myxococcaceae* Jahn
  - Genus I. *Myxococcus* Thaxter
  - Genus II. *Chrondrococcus* Jahn
  - Genus III. *Angiococcus* Jahn
  - Genus IV. *Sporocytophaga* Stanier

ORDER V. SPIROCHAETALES Buchanan

- Family I. *Spirochaetaceae* Swellengrebel
  - Genus I. *Spirochaeta* Ehrenberg
  - Genus II. *Saprospira* Gross
  - Genus III. *Cristispira* Gross
- Family II. *Treponemataceae* Schaudinn
  - Genus I. *Borrelia* Swellengrebel
  - Genus II. *Treponema* Schaudinn
  - Genus III. *Leptospira* Noguchi

Supplement I

ORDER RICKETTSIALES Gieszczykiewicz

- Family I. *Rickettsiaceae* Pinkerton
  - Genus I. *Rickettsia* da Rocha-Lima
  - Genus II. *Coxiella* Bengtson
  - Genus III. *Cowdria* Bengtson
- Family II. *Bartonellaceae* Gieszczykiewicz
  - Genus I. *Bartonella* Strong, Tyzzer and Sellards
  - Genus II. *Haemobartonella* Tyzzer and Weinman
  - Genus III. *Grahamella* Brumpt
  - Genus IV. *Eperythrozoon* Schilling
- Family III. *Chlamydozoaceae* Moshkovsky
  - Genus I. *Chlamydozoon* Halberstaedter and von Prowazek
  - Genus II. *Miyagawanella* Brumpt
  - Genus III. *Colesiota* Rake
  - Appendix: Genus A. *Caryococcus* Dangeard
    - Genus B. *Drepanospira* Petschenko
    - Genus C. *Holospora* Haffkine

Supplement II

ORDER VIRALES Breed, Murray and Hitchens

SUBORDER I. PHAGINEAE Holmes

- Family I. *Phagaceae* Holmes
  - Genus I. *Phagus* Holmes

SUBORDER II. PHYTOPHAGINEAE Holmes

- Family I. *Chlorogenaceae* Holmes
  - Genus I. *Chlorogenus* Holmes
  - Genus II. *Carpophthora* McKinney
  - Genus III. *Morsus* Holmes
  - Genus IV. *Aureogenus* Black
  - Genus V. *Galla* Holmes
  - Genus VI. *Fractilinea* McKinney

Family II. *Marmoraceae* Holmes
Genus I. *Marmor* Holmes
Genus II. *Acrogenus* Holmes
Genus III. *Corium* Holmes
Genus IV. *Nanus* Holmes
Genus V. *Rimocortius* Milbrath and Zeller
Genus VI. *Adelonosus* Brierley and Smith
Family III. *Annulaceae* Holmes
Genus I. *Annulus* Holmes
Family IV. *Rugaceae* Holmes
Genus I. *Ruga* Holmes
Family V. *Savoiaceae* Holmes
Genus I. *Savoia* Holmes
Family VI. *Lethaceae* Holmes
Genus I. *Lethum* Holmes
Suborder III. Zoophagineae Holmes
Family I. *Borrelinaceae* Holmes
Genus I. *Borrelina* Paillot
Genus II. *Morator* Holmes
Family II. *Borreliotaceae* Holmes
Genus I. *Borreliota* Goodpasture
Genus II. *Briareus* Holmes
Genus III. *Scelus* Holmes
Genus IV. *Hostis* Holmes
Genus V. *Molitor* Holmes
Family III. *Erronaceae* Holmes
Genus I. *Erro* Holmes
Genus II. *Legio* Holmes
Genus III. *Formido* Holmes
Family IV. *Charonaceae* Holmes
Genus I. *Charon* Holmes
Genus II. *Tarpeia* Holmes
Genus III. *Tortor* Holmes
Family V. *Trifuraceae* Holmes
Genus I. *Trifur* Holmes
Family VI. *Rabulaceae* Holmes
Genus I. *Rabula* Holmes

Supplement III
Family I. *Borrelomycetaceae* Turner
Genus I. *Asterococcus* Borrel et al.

Species and Genus. In bacteriology the terms species and genus are used but the concept of these is somewhat vague as there is little knowledge of genetics in relation to bacteria. In bacteriology, a species is theoretically a single kind of bacterium, all individual cells of which are identical or nearly so. In actuality this identity of cells rarely exists. In any culture of a given species cells may be found which, while having the outward form, staining properties and other characters of most of the cells in the group, possess different metabolic properties, different antigenic composition and so on. Usually these differences are not extreme and may represent only temporary fluctuations from the principal type. When two bacteria have one or more *well-marked* morphological, metabolic or other differences between them which are *constant,* the two may be regarded as distinct species. Here we run into an arbitrary difficulty. Who is to determine what character or characters shall constitute "well-marked," and "sufficient" distinction to be the basis for a differentiation between species or genera? The same differences may be used as a basis of generic or tribal or even familial distinction between some other kinds of bacteria. Bacterial species, therefore, are rather illy defined.

The concept of genera is, in many instances, equally nebulous. A genus is theoretically, and ideally, a group of species all of which bear sufficient resemblance to one another to be considered closely related and easily distinguishable from members of other groups or genera. The boundaries of some genera are sharply defined by as few as three characteristics, as in the genus *Bacillus:* (1) aerobic; (2) spore-forming; (3) rods. The boundaries of other genera are sometimes more difficult of definition; for example, the genera *Salmonella, Escherichia, Shigella,* and *Aerobacter,* all of which are non-sporeforming, gram-negative, facultative, aerobic rods. An organism of one genus may thus possess several of the important (?) characters of two or three or more other genera and its proper allocation to one of these is often difficult and must be decided on an arbitrary basis.

Bacterial Nomenclature. The selection of the proper name for an organism requires a knowledge of the rules of nomenclature, of previously used names, and an exhaustive knowledge of the characteristics of the organism and of similar organisms already named. The name of an organism is (or should be) a descriptive symbol. It should convey a definite idea of the organism named. This saves words and time. But it requires meticulous care not to use the same name for different organisms, and fully and accurately to describe the organism itself. Unfortunately, there are many violations of these rules.

In naming a bacterium, certain definite conventions are followed. Each species is allowed a "first" and "last" name only. This two-name scheme is called the binominal system and originated in 1760 under the leadership of Linnaeus. The first name of a bacterium refers to the genus, and is usually a Latin or latinized word (sometimes Greek), generally a noun, based on the morphology of the organism or on the name of the discoverer. It is written with a capital letter. The last name is the species name and is usually descriptive of the noun, referring to its color, source, disease production, discoverer or some other distinguishing point. It is not capitalized. Genus and species names are generally italicized. For example, the name *Bacillus anthracis* indicates that the organism is a spore-bearing, aerobic rod (properties of the genus *Bacillus*), while *anthracis* calls attention to the fact that this species of the genus *Bacillus* produces the disease anthrax. The name *Spirillum rubrum* shows that the organism is a true saprophytic bacterium, rigid, spiral in structure, non-sporeforming, motile, and gram-negative (all properties of the genus *Spirillum*), and that the species named is characterized by a red color (rubrum). The name *Clostridium novyi* indicates a gram-positive, spore-forming, rod-shaped organism, saprophytic or parasitic, and restricted to growth in the total absence of *free oxygen*. These are properties of the genus *Clostridium*. This particular species bears the name *novyi* in honor of Dr. F. G. Novy of the University of Michigan, who discovered the organism and its relation to a disease, gas gangrene. The practice of using personal names for newly discovered species of bacteria is obsolescent, although many generic names are derived from the discoverers or original students of the genus; for example the genus *Salmonella*, from a famous American bacteriologist named Salmon.

In writing of bacteria, it is customary to abbreviate the generic name, using only the initial letter if it is clear what genus is meant; for example *B. anthracis* for *Bacillus anthracis*. Sometimes the abbreviation may be longer, as *Br. abortus* for *Brucella abortus*. Medical bacteriologists often dispense with these rules and designate organisms by the disease with which they are most frequently associated, for example, meningococcus, pneumococcus, typhoid bacillus, etc. This is a convenient but loose custom not in accord with rules of nomenclature.

The entire system of bacterial nomenclature, its rules, underlying philosophy and methods were expertly drawn up in an International Code by Buchanan, St. John-Brooks and Breed and an International Judicial Commission at Copenhagen in 1947. This should be consulted for full details.[7]

Biochemistry and Taxonomy. With the increase in knowledge of chemistry in general, there came an increase in knowledge of the biochemistry of bacteria. When Koch showed the way to pure culture study, it became possible to differentiate between morphologically identical organisms by their biochemical properties such as ability to ferment certain sugars, and so on. These properties soon found their way into taxonomy as the bases of subdivisions of previously large and heterogeneous groups. Today many distinctions are based on biochemical properties of morphologically indistinguishable organisms.

As mentioned before, variation is a disturbing factor. For example, by cultivating a number of bacteria (e.g., *Escherichia* and *Aerobacter*) normally not fermenting salicin, in medium containing this glucoside for long periods, salicin-fermenting variants have been induced.[8] According to older systems of classification, these variants would have been regarded as other "species." In these experiments *Esch. anindolica* became *E. coli communior, E. formica* changed to *E. coli,* and *E. gruenthali* became *E. paragruenthali.* Modern keys recognize these organisms as variants of *E. coli.*

The danger in establishing species and genera on the basis of single or only a few characters which may be subect to variation is thus clearly shown. The larger the number of characters on which species and generic and other differences are based, the less confusion will result because, while individual characters may vary, several characters rarely or never change all at once under ordinary circumstances. The basis of family, tribe, genus and species distinctions would be greatly broadened and stabilized if the distinctions rested on successively greater numbers of, and more stable, characters.

Antigenic Structure and Taxonomy. In attempts to introduce still greater accuracy into systems of classification, immunology has been drawn upon, and minute antigenic differences* between organisms formerly believed to be identical are now often used to formulate new groupings. For example, serological subdivisions of a group are designated as *types,* like pneumococcus types I, II, III, etc., or *groups,* as Lancefield group A, B, C, etc., of streptococci.

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* Differences in protein or carbohydrate composition or surface structures.

Chapter 6

ENVIRONMENTAL FACTORS IN MICROBIOLOGY

Life, in the many animal and plant forms which we see today, reflects natural selection and adaptation of protoplasm to existence under a great variety of conditions. As a result of evolutionary processes microorganisms have appeared, capable of utilizing what seem to us the most indigestible of foods (pure sulfur, kerosene, carbolic acid, etc.), and thriving in the (to us) most remote, dismal and uninhabitable places, such as the depths of ocean ooze, subterranean slimes, petroleum wells, hot sulfur springs and arctic seas, as well as inside animal intestines, and in the tissues of animals and plants. So complete and highly specialized is the adaptation that many forms of microorganisms perish when transferred suddenly to other environments. In this chapter we shall discuss factors in the environment which affect microorganisms.

Temperature. One of the most important factors influencing all forms of life is temperature.[1] *It is of basic significance* that the optimal and limiting temperatures of microorganisms, and indeed most living cells, are the optimal and limiting temperatures of *enzymes* (see page 265).

With respect to mere *survival* of heat, most microorganisms in an *actively growing* (vegetative) state are readily killed by exposures to temperatures of around 70° C. for 1 to 15 minutes. Boiling kills them in a few moments. Thermophils* are more resistant, some vegetative cells surviving 80 to 90° C. for many minutes. Bacterial spores are still more resistant, as has already been pointed out. Spores of thermophils may survive boiling or higher temperatures for many hours.

The relations of temperature to the *growth* of bacteria are, however, somewhat more complex. Many species indigenous to the soil, water, and body grow well at temperatures from 25° to 40° C. (human body temperature is 37° C.). Species growing well at such temperatures are called *mesophilic* (moderation-loving). Some of the soil and water bacteria grow best at temperatures very little above the freezing point (4° to 10° C.) and these are referred to as *cryophilic* or *psychrophilic* (cold-loving). Certain marine forms, adapted to life at around 4° C., will die if held above about 30° C. for more than a few minutes.[2] These would be called *obligate psychrophils, i.e.*, they are limited to low temperature as a condition of life. There are, in addition, several species of bacteria which thrive only at high temperatures (60° to 80° C.). Such species are called *thermophilic* (heat-loving). Some species of thermophilic bacteria are found in hot sulfur springs while others occur in milk, soil, manure, and related situations.

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* Organisms preferring temperatures higher than about 45° C.

A distinction should be made in all cases between ability to *endure* a given temperature and ability to *grow well* under the same conditions. Many bacteria, for example, can *live* for months or years at freezing, yet do not *grow* at all.[3] *Thermoduric* organisms *survive* well at higher temperatures, but only *thermophilic* species *grow* well under such conditions.

The bacteria which cause disease in warm-blooded animals are adapted to grow best at body temperature (37° C.) and medical bacteriology, therefore, deals chiefly with mesophilic bacteria. Most of the bacteria active in the "spoiling" of vegetables, the decay of meat and the souring of milk are also mesophilic and grow very slowly, if at all, at refrigerator temperatures (4° to 10° C.). For this reason, foods may be preserved for days or weeks if properly refrigerated. However, there are some psychrophilic species which cause spoilage of food stored in refrigerators. Refrigerators should be frequently cleaned and aired to discourage psychrophilic molds and bacteria. Some asporogenous (non-sporeforming) bacteria are definitely more heat-resistant than others. Thus, asporogenous thermophils may thrive at 60° or 65° C., a temperature which soon kills pathogenic varieties.

Thermal Death Point. The temperature at which all the bacteria of a given species are killed after ten minutes' exposure is spoken of as the "thermal death point" of that species. This is a very inexact expression, because at a given temperature, say 70° C., the bacteria do not all die simultaneously and suddenly just as the clock registers the expiration of ten minutes. "Thermal death point" merely tells us when the *last survivor* of all has expired, the number of live cells having been decreasing continuously during the exposure period. In measuring thermal death point much depends on the species and numbers of bacteria originally present, the age of the bacteria, the acidity of the suspending fluid, its osmotic pressure and its composition, etc., so that the term "thermal death point" can be correctly used only if the exact conditions of a given experiment are known. However, for certain purposes, the information is of practical use, as in the preservation of certain foods, milk, etc.

Thermal Death Time. This is the length of time required to kill all of the bacteria in a given substance at a stated temperature. The determination of thermal death time is subject to much the same errors as the measurement of thermal death point, but under known conditions is of practical use. Thus, in pasteurizing, milk is heated to 62° C. and held there for thirty minutes. This is considerably in excess of the *thermal death time, at 62° C., in market milk,* of all the disease bacteria known to be transmitted by milk. Many nonpathogenic bacteria, both sporogenous and asporogenous, survive pasteurization so that pasteurized milk is safe but not necessarily sterile.

Rate of Death. As noted above, death of all the bacteria in a given material does not occur simultaneously unless the lethal influence be overwhelming. On the contrary, death occurs in a definite relationship to time, the rate being determined by various factors such as temperature, moisture, acidity, etc.

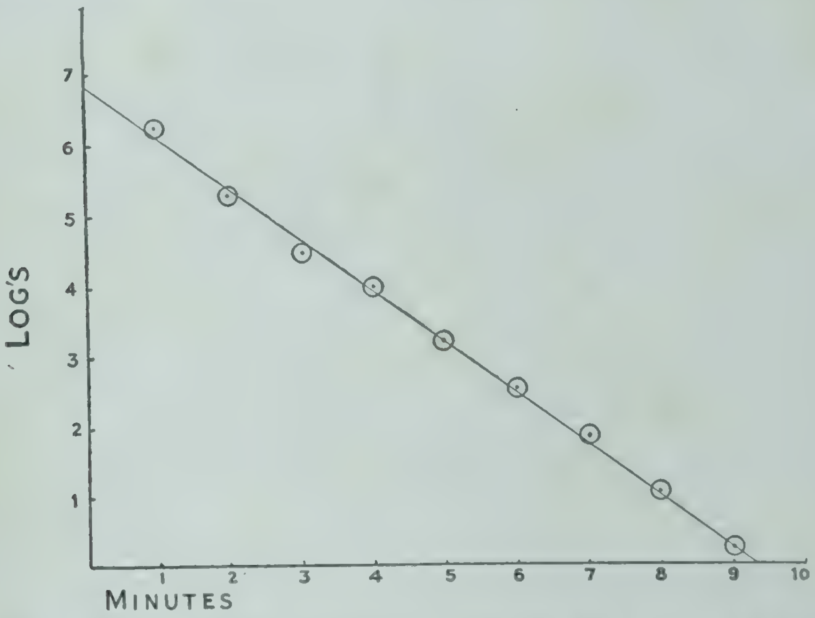

Fig. 61. Relation between numbers of organisms and time in a 1 per cent solution of phenol containing typhoid bacilli. The logarithms are of numbers of bacilli per ml. The straight-line curve is characteristic of the relation between time and survivors under any definitely adverse conditions when no growth occurs.

Older cells are more resistant than young ones. As will be shown in Chapter 10, moisture and acidity increase the vulnerability of cells to heat. Most killing processes behave like a monomolecular chemical reaction, progressing in accord with the laws governing mass action. If the numbers of organisms surviving in a disinfection test be determined at various intervals, and plotted against the intervals, regular curves are formed. If the *logarithms* of the numbers be similarly plotted, a straight line is formed (Fig. 61).

Hydrogen Ion Concentration. Another physical factor profoundly affecting bacteria is the acidity or alkalinity of the fluid in which they are suspended. This is usually expressed in terms of *hydrogen ion concentration* since, as the student will recall from his studies of chemistry and physics, it is the concentration only of *dissociated* or *ionized* hydrogen (or hydroxyl) that determines the actual, *effective acidity* (or alkalinity) of a given solution. Unfavorable influences of many sorts may be much enhanced in fluids having an acid reaction. For example, coagulation by heat occurs more readily in acid solutions. Thus, milk which is only very slightly sour may curdle on being warmed. The close interrelationship of temperature and acidity has been shown also by Jordan and Jacobs.[3a]

The reaction of bacteriological culture media must be very carefully adjusted, with respect to *hydrogen ion concentration,* the degree of acidity depending on the bacteria to be cultivated.

Titrable Acidity and Hydrogen Ion Concentration. In order to avoid complicating this discussion the new concept of the formation of hydronium ions $(H_3O)^+$ in the dissociation of water is omitted. Suffice it to say that according to this idea the hydronium ion may be regarded as composed of one undissociated water molecule plus one hydrogen ion: $H_2O + H^+ \rightarrow (OH_3)^+$.* Since the traditional ideas of electrolytic dissociation[4] still provide a useful basis for discussion of acidity they will be followed here.

Students familiar with those theories will remember that, according to them, strong acids are those which, when dissolved in water, dissociate largely into positively charged hydrogen ions and a negatively charged ion. For example, each molecule of sulfuric acid dissociates into 2 hydrogen ions and a sulfate ion. Weak acids like acetic or citric also dissociate, but to a much lesser degree. The student will remember that the acidic *activity* of any acid solution depends upon the concentration of these ions of hydrogen and this is obviously dependent upon the ability of the acid to give them off into the solution or to dissociate. Thus, two acid solutions may be of the same concentration with respect to the *total* amounts of hydrogen available, yet have widely differing activities due to differences in the amount of active or *ionized* or *dissociated* hydrogen. Here we deal with a *capacity* effect, *i.e.,* total available (dissociated plus undissociated) acid, and an *intensity* or activity effect (dissociated acid or hydrogen ions alone).

As an example, let us consider acetic acid (vinegar acid) and hydrochloric acid. A liter of a normal solution of each contains exactly 1 gm. of total *available* hydrogen, yet the *activity* of the N/1 acetic acid is slight while that of the N/1 hydrochloric acid is great. Of the gram of available hydrogen in the acetic acid solution only about 1.36 per cent is in an ionized state, so that there is, in the liter of solution, only 0.0136 gm. of hydrogen ions.† The gram of hydrogen in the liter of N/1 HCl solution is about 91.4 per cent ionized, giving 0.914 gm. of hydrogen ions per liter.* The N/1 HCl, therefore, is about 67 times as active or "strong" as the N/1 acetic acid.

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* Free hydrogen ions do not actually exist, since hydrogen is a proton; but they enter water molecules, forming hydronium ions.

† $\frac{N1}{0.0136} = N/73.5$; $\log 1/73.5 = -1.86$ or *p*H 1.86

If one were to titrate the solutions, *i.e.*, add N/1 NaOH solution until each became neutral, the *total* amount of alkali required would be exactly the same in each case. This is due to the fact that, as the alkali combines with the hydrogen ions, more hydrogen ions take their place from the undissociated acid which strives to maintain a constant hydrogen ion concentration consistent with its dissociation constant. Each acid finally gives up all its available hydrogen and, since each of the solutions by definition (N/1) contained exactly 1 gm. of available hydrogen to start with, each requires the same amount of alkali for its neutralization.

TABLE I

RELATIONSHIPS OF HYDROGEN ION CONCENTRATIONS EXPRESSED IN VARIOUS WAYS

Reaction	Fraction of Normality*	Hydrogen Ions per Liter†	Logarithms of H Ion Concentrations	Expressed as *p*H
Acid	*N*/1	1.0	− 0	0.0
Acid	*N*/10	0.1	− 1	1.0
Acid	*N*/100	0.01	− 2	2.0
Acid	*N*/1,000	0.001	− 3	3.0
Acid	*N*/10,000	0.000,1	− 4	4.0
Acid	*N*/100,000	0.000,01	− 5	5.0
Acid	*N*/1,000,000	0.000,001	− 6	6.0
Neutral ...	Pure water	0.000,000,1	− 7	7.0
Alkaline ...	*N*/1,000,000	0.000,000,01	− 8	8.0
Alkaline ...	*N*/100,000	0.000,000,001	− 9	9.0
Alkaline ...	*N*/10,000	0.000,000,000,1	−10	10.0
Alkaline ...	*N*/1,000	0.000,000,000,01	−11	11.0
Alkaline ...	*N*/100	0.000,000,000,001	−12	12.0
Alkaline ...	*N*/10	0.000,000,000,000,1	−13	13.0
Alkaline ...	*N*/1	0.000,000,000,000,01	−14	14.0

* With respect to hydrogen or oxygen-hydrogen *ions*.

† Grams.

A measurement of hydrogen ion concentration, therefore, differs from a titration, in that the former determines only the actual concentration of *ionized* or *active* hydrogen at the moment, without calling out any of the reserve, undissociated acid; while the latter measures the total, available, *ionizable* as well as *ionized* hydrogen, and calls upon all the reserve, undissociated acid until it is exhausted and the solution has no acidic property left, or is neutral.

The term "normal" generally refers to the presence of 1 gm. of *total available* hydrogen per liter of solution. It cannot, therefore, be properly used in speaking of *active* hydrogen (H ions) unless the phrase "with respect to hydrogen ion concentration" be appended. Thus, a solution normal *with respect to hydrogen ion concentration* contains 1 gm. of *hydrogen ions* per liter, while a tenth normal (N/10) solution (*with respect to hydrogen ions*) contains 0.1 gm. of *hydrogen ions* per liter, and so on (see Table I).

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* $\frac{\text{N1}}{0.914} = \text{N}/1.094$; Log $1/1.094 = 0.04$ or pH 0.04

Method of Expressing Hydrogen Ion Concentration. Water ionizes reversibly to a very slight degree (about 1 molecule in 555 millions): $H_2O \rightleftarrows H^+ + OH^-$. Since each dissociated molecule of water yields one H^+ ion and one OH^- ion, these are present in equal concentrations in pure neutral water. The relation between the dissociated and undissociated water is a constant. According to the law of mass action this constant (K) is the product of: $[H^+]$* by $[OH^-]$ divided by $[H_2O]$.

$$\frac{[H^+] \cdot [OH^-]}{[H_2O]} = K.$$

Since $[H_2O]$ is so very large, possible variations in $[H^+] \cdot [OH^-]$ affect K very slightly and $[H_2O]$ is therefore generally considered as 1. The formula is therefore written as $[H^+] \cdot [OH^-] = K$. Now, by conductivity measurements, K of pure neutral water has been found to equal, at 22° C., $\frac{1}{100{,}000{,}000{,}000{,}000}$ or 1×10^{-14}; thus, $[H^+] \cdot [OH^-] = 10^{-14}$. Since $[H^+]$ and $[OH^-]$ are equal in neutral water, then $[H^+] = 10^{-7}$ and $[OH^-] = 10^{-7}$. These expressions can be simplified by using the notation of Sörenson, which substitutes *p*H for $[H^+]$ and a positive value for the negative logarithm. Thus, in a neutral solution, *p*H = 7.0.

Since the number representing *p*H is derived from the logarithm of a fraction, it is obvious that the larger the fraction the smaller will be the number in its logarithm. Therefore, numbers below 7 represent greater hydrogen ion concentrations or acidities, while numbers above 7 represent lower hydrogen ion concentrations or alkalinities. Table I shows some of these relationships.

It is important for the microbiologist to remember that, as a rule, increases in temperature cause increases in dissociation so that a solution which is neutral or slightly alkaline at room temperature (about 22° C.) may become definitely acid at incubator temperature (37° C.). If a solution is prepared at a definite *p*H while near the boiling point, it will be more alkaline when cool. It is also important to note that a change in *p*H equivalent to 1 on the *p*H scale, represents a 10-fold change in hydrogen ion concentration, while a change from *p*H 7.0 to 7.3 represents a 2-fold increase.

The *p*H of Culture Media. For measuring *p*H certain dyes are often used which take a definite color at given acidities or alkalinities. A dye like phenol red may be selected. This has a canary yellow color at *p*H 7.0 or below, a slightly orange or pinkish-yellow color at *p*H 7.4, is definitely red-orange at pH 7.6 and deep magenta at pH 8.0 or above. Many other dyes of various colors indicating ranges of pH from 1 to 12 are available. Some of the common pH indicators are listed in Table II.

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* The brackets are the conventional sign for "concentration of." Some prefer the concept of activity rather than concentration.

TABLE II

SOLUTIONS FOR TESTING pH AND ADDING TO FERMENTATION MEDIA

Indicator	pH Range	Concentration gm./100 ml. 95% alcohol*	Color		For Addition to Media
			full acid	full alkaline	
Thymol blue	1.2–2.8	0.04	red	yellow	
Brom phenol blue	3.0–4.6	0.04	yellow	blue	
Methyl red†	4.4–6.0	0.02	red	yellow	
Brom cresol purple	5.2–6.8	0.04	yellow	purple	1.6 gm./100 ml.alc. Use 1 ml. in 1 liter of medium
Brom thymol blue	6.0–7.6	0.02	yellow	red	0.02 gm./100 ml. alc. Use 1 ml. sat. sol. per liter
Phenol red	6.6–8.2	0.04	yellow	blue	
Wide Range Mixture					
Brom cresol purple—phenol red	5.2–8.2	sat. aqueous solution	yellow	red	Use 1 ml. each per liter of medium adjusted to pH 7.0

* Commercial alcohol is usually acid. Alcoholic solutions of indicators, therefore, take their acid color. In each case add NaOH (N/20) until an intermediate tint of the whole solution is obtained. Equilibrium is sometimes reached slowly and the alkali must therefore be added cautiously and given time to react.

† Methyl red is reduced by many bacteria and is therefore added only *after* incubation of a culture. Some organisms are adversely affected by certain indicators; e.g., *C. diphtheriae* by phenol red.

*Measuring pH Colorimetrically.** Five-tenths ml. of a 0.02 per cent alcoholic solution of phenol red, added to 5 ml. of freshly prepared broth, usually turns a canary yellow color. This indicates a neutral or acid reaction but, as phenol red reaches its maximum yellow color at pH 7 (neutral), we have no way of knowing whether the medium is *exactly neutral* or, if acid, how great a degree of acidity the medium really has. We therefore discard the first test, and select a dye which changes color in a more acid range. For this purpose we may select brom cresol purple, having a *p*H range from 5.2 (canary yellow) to 6.8 (purple). The test is repeated with this dye and we often find that in the fresh medium this indicator turns a reddish-brown, indicating a *p*H of around 6.0, definitely acid.

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* Because of the perfection and simplification of electrical instruments for measuring pH, the colorimetric method is now obsolescent. However, it is still widely used in certain types of work.

In order to determine this exactly (if desired) a comparator block (Fig. 62) may be used, following the scheme outlined below. Tube 1 contains 5 ml. of a solution of *known* *p*H and 0.5 ml. of the selected dye. Tube 2 contains 5 ml. of medium, but no dye. Tube 3 contains 5 ml. of medium with 0.5 ml. of the same dye. Tube 4 contains water.

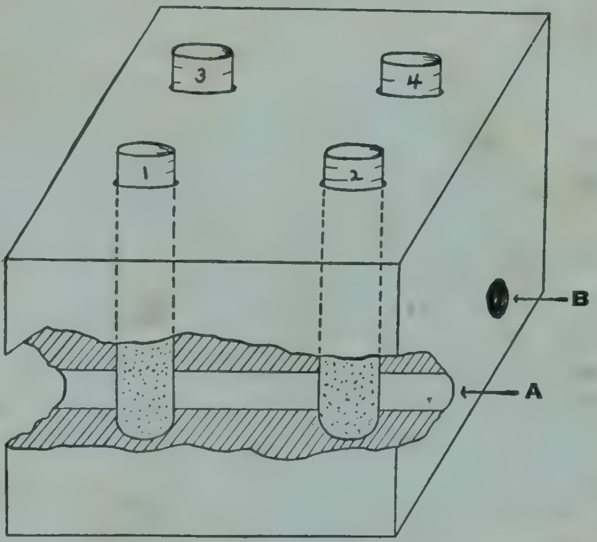

Fig. 62. Simple apparatus for determining and adjusting *p*H. For explanation see text.

Different solutions of *known* *p*H (with the dye) are substituted for tube 1 till the color seen on looking through opening A exactly matches that seen in opening B. We can then state that the *p*H of the medium is the same as that of the *known* solution in tube 1.

Tube 2 is included in order to superimpose the natural yellowish tint of the fresh medium on the color of the dye itself in tube 1, since tube 3 also contains the color due to the dye plus the natural color of the medium. Tube 4 is included to balance the optical equation in A and B.

There are many modifications of this method. For example, tube 1 with its known *p*H color may be replaced, in an appropriate apparatus, by pieces of colored glass.

Adjustment of the pH. By adding N/20 sodium hydroxide from a buret to tube 3, a drop at a time, we observe that, as each drop is added, the color changes from yellow toward yellow-orange, then to red-orange. Enough hydroxide is finally added so that the color matches that of the *p*H 7.4* standard; or it can be made to match any other standard which we adopt.† The amount of N/20 alkali used to adjust the reaction of the 5 ml. is now noted and the amount necessary to adjust the remaining bulk of the medium is computed. This may be found to be one fourth of the total volume or more. In order to avoid dilution of the medium by the addition of so large an amount of fluid, we substitute N/1 sodium hydroxide in place of the N/20 alkali, using one twentieth of the calculated amount.

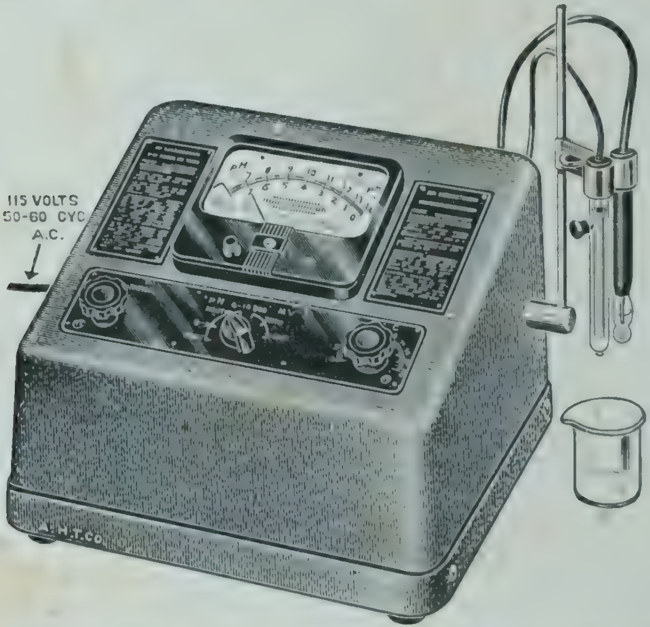

Fig. 63. A modern type of apparatus (*p*H meter) for determining the hydrogen ion concentration of fluids. The fluid to be tested is placed in the small beaker, the 2 electrodes are lowered into it, and the knobs on the machine adjusted till the *p*H is readable on the scale.

After adding this amount of alkali the reaction of the whole is tested again to verify the correctness of the procedure. The mixture is then boiled for ten minutes to hasten the formation of precipitates due to the addition of the alkali. Loss of water by evaporation should be compensated for. It may be determined by weighing the vessel of medium before and after boiling. The fluid is centrifuged or passed through a folded filter paper or cotton and gauze to remove any solid matter and may be collected in test tubes or flasks with cotton plugs. It is then sterilized, since, as the early students of spontaneous generation noted, it is a "putrescible fluid" and would soon teem with "animalcules" if not heated thoroughly. The final *p*H of the finished product should be checked again, as it tends to change somewhat on heating.

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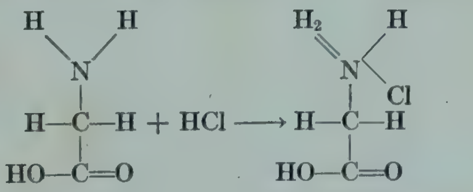
* This is a favorable *p*H for a wide variety of bacteria.

† More conveniently, the titration and adjustment may be done using the whole volume and the electrometric "*p*H Meter."

Buffers and Buffer Action. In the ordinary titration of, let us say, N/10 acid with N/10 alkali, in distilled water using phenol red as indicator, there comes a point in the titration when a single drop of N/10 alkali from the buret changes the reaction from distinctly acid (yellow; *p*H more acid than 6.5) to definitely alkaline (*p*H more alkaline than 8 or 9) as evidenced by the appearance of a deep magenta color. Thus a single drop of N/10 alkali (or acid) produces a very large alteration in *p*H. In attempting an adjustment of the *p*H of bacteriological media such as infusion broth it is found that, unlike the titration of the aqueous solutions of acid or alkali, *no sharp end point is reached,* and that the change from an acid to an alkaline reaction or vice versa is very gradual, requiring the continuous addition of relatively large amounts of acid or alkali. In other words, even at or near the neutral point, the solution being titrated shows a marked tendency to resist any change in its reaction. This is due to what is known as "buffer action" on the part of certain constituents of the broth. These substances combine with the acid or alkali as fast as it is added and so prevent change of reaction of the medium until they are all used up.

There are several sorts of substance which have the property of acting as buffers. Important among these, from a biological standpoint, are amino acids and their compounds, the polypeptides, proteoses, proteins, etc. These are neither strongly acid nor alkaline but will combine with either acid or alkali.[4, 5]

To cite a simple example of buffer action by an amino acid, we may note that glycine combines with HCl or NaOH as follows:



Glycine *Glycine hydrochloride*

or,

$$CH_2{-}NH_2{-}COOH + NaOH \longrightarrow CH_2{-}NH_2{-}COONa + H_2O.$$

Sodium glycinate

Other important buffers are salts of weak acids, as acetates or phosphates. A mixture of sodium acetate and acetic acid is a good buffer mixture, since the acetate dissociates largely and the acetic acid slightly. If HCl is added, the H^+ from the HCl and from the acetic acid together exceed the dissociation constant of both acids and enough of each acid reassociates to reduce the amount of free hydrogen ions to the initial *p*H. If the concentration of H^+ still exceeds the dissociation constants of the acids, then the excess H^+ from the HCl combines with acetate ions forming undissociated acetic acid and NaCl. A reverse process occurs on the addition of alkali such as NaOH. Sodium acetate and water are formed by reaction with the acetic acid and, if this still leaves free OH^-, undissociated acetic acid will dissociate to liberate H^+ which combines with the OH^-, thus maintaining the initial *p*H until all the acetic acid is used up.

One of the most commonly used buffers is a mixture of the monobasic and dibasic phosphates, KH_2PO_4 and K_2HPO_4. These dissociate relatively little. When acid is added the dibasic salt absorbs H^+, changing into the monobasic and forming a potassium salt with the acid. When alkali is added, the monobasic salt gives up H^+ to form H_2O with the OH^-, and the incomplete salt combines with the cation of the alkali.

Other important buffer constituents of organic media are carbonates and bicarbonates:

$$Na_2CO_3 + 2HCl \longrightarrow 2NaCl + H_2O + \overset{\textit{gas}\ \nearrow}{\boxed{CO_2}}$$

$$NaHCO_3 + NaOH \longrightarrow Na_2CO_3 + H_2O.$$

Lumps of marble ($CaCO_3$) are sometimes added to cultures to maintain the *p*H unchanged, that is, to neutralize the acid produced by fermentation. Colloidal substances such as charcoal, precipitates of phosphates, kaolin, etc., which absorb H or OH ions directly, are also active buffers and are used in biological systems. Most microorganisms, like bacteria, grow best in fluids buffered within the range of from *p*H 6.0 to 8.0, or at *p*H 7.4. Molds and yeasts prefer *p*H around 6.0.

Other Ions. The hydrogen and hydroxyl ions are not by any means the only ones having a marked influence on microorganisms. The well-being and life of all cells and tissues is in great part dependent on the absence of or presence, and suitable concentration, of a multitude of ions of different sorts. In the chapters on disinfection and cultivation of microorganisms some practical applications of our knowledge of favorable and unfavorable ions are described. Here we may outline general principles underlying those applications.

First, it is essential to remember that in attempting to study the effects of a given kind of ion in any physiological fluid, such as laboratory culture media, blood, etc., the results are influenced by the presence of other ions, molecules and colloidal particles, and other factors, some very difficult to control. Even in a simple aqueous solution of sodium chloride, we have to consider the effect of

1. the cation (Na^+) per se
2. the anion (Cl^-) per se
3. the undissociated molecules ($NaCl$)
4. the H and OH ions of the water
5. the species of microorganism and its recent cultural history
6. solution of gases from the air and of impurities from the walls of the tubes or flasks
7. temperature
8. others.

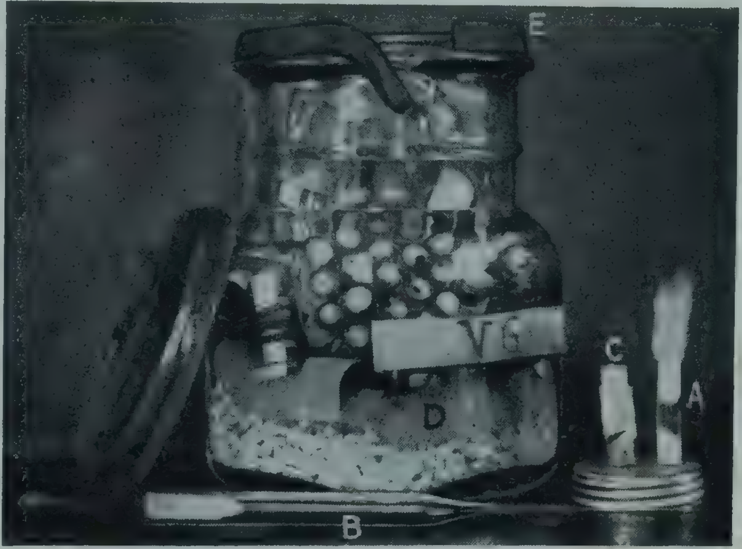


Fig. 64. *A,* Sterile vial; *B,* pipette; *C,* blooded vial; *D,* cotton; *E,* plasticine. (From Frobisher, Parsons, Pai, and Hakim, in J. of Lab. & Clin. Med., vol. 32.)

The effects of most of these are fairly constant or may be equalized in comparative experiments, but some (especially 5 and to some extent 6) have an unknown or varying value. In work involving artificial culture media such as meat or vegetable infusions and other organic mixtures of unknown composition, the variables and extraneous factors are numerous. Combinations between the organic matter and the ions, effects of the ionized substance, especially organic acids and their salts on surface tension, all have to be considered. It is clear that an *exact* explanation of the effects on microorganisms of various ionizing substances is difficult if not impossible. However, a large number of careful investigations have been made which, while not furnishing complete explanations, have yielded many valuable and useful facts that permit certain generalizations.

Influence of Cations. Many inorganic substances, as well as organic compounds which ionize, are physiologically effective, favorably or unfavorably, in extremely small concentrations. These substances may first be considered with respect to the toxicity of their cations. In general, salts of the heavy metals are more toxic to bacteria than those of the light metals. Monovalent metals are usually less toxic than multivalent metals. The toxicity of most salts is reduced by the presence of protein, which reduces the various ion concentrations by physico-chemical combinations. Other things being equal we may say that chlorides of the alkaline earth metals (Na, K, Li, Sr, Mg, Ca) and NH_4 are relatively harmless, and even desirable and stimulating to most bacteria in the order named, with KCl and NaCl usually harmless in concentrations as high as 1.5 per cent and not exerting very striking effects on many bacteria, after adaptation to artificial cultivation, even in concentrations of 4 or 5 per cent. In general, chlorides of the heavy metals (Zn, Cu, Fe, Pb, Hg, etc.), while slightly stimulating in *extremely* low concentrations (*e.g.*, Hg : 0.000,005M), exert definitely harmful effects if present in greater amounts than about 0.007 per cent in the case of mercury, or 0.008 per cent when the metal is lead.

The less toxic salts act favorably in a great variety of ways, sometimes by effectively suppressing the ionization of unfavorable substances, or by reacting with these substances so as to prevent them from affecting bacteria unfavorably. For example, the presence of calcium chloride might be very effective in reducing the toxicity of sodium oxalate by forming an insoluble precipitate of calcium oxalate, thus removing the toxic oxalate radicle from the solution. Many salts probably act also by altering the permeability of the cell membrane and by affecting its electrical charge, enzymes, etc. The importance of this action in metabolism and immunology is discussed in the chapters on those topics. Salts also probably act by forming chemical compounds with constituents of the cell substances; whether favorable or unfavorable compounds depends on the salt and conditions.

The toxicity of alkalis varies much, as does the toxicity of inorganic acids, but it depends mainly on the concentration of hydroxyl ions (pOH). That the cation plays its role in alkalies, as it does in acids and salts, however, is indicated by the fact that barium hydroxide, a much weaker (less dissociated) alkali than sodium hydroxide, is nevertheless much more toxic at the same concentration of hydroxyl ions. This is partly because the barium ion is more toxic than the sodium ion.

Influence of Anions. In addition to the cation of a salt, its desirability or toxicity from the standpoint of bacteria is determined in part by the anion. In order of favorable action with regard to bacteria (especially *E. coli*) we may list several anions (with sodium as the cation) as follows: sulfate, tartrate, chloride, nitrate, acetate, citrate, oxalate, iodide, benzoate, salicylate, tellurate; sulfates the least toxic, tellurates the most unfavorable. There are data on many others intermediate between these.[6]

Many of the experiments on salt effects have been made with *Escherichia coli* as a test organism. This has provided much valuable information but it is very important to remember that species vary greatly in respect to sensitivity toward various ions. For example, *C. diphtheriae* grows well in the presence of tellurites, which are toxic to many species; *B. anthracis* grows in the presence of oxalates, toxic to other organisms, and so on. The toxicity of different anions is illustrated by the effects of sodium chloride, sodium benzoate, and sodium salicylate. Sodium chloride is a desirable component of nutrient media. Sodium benzoate and salicylate are bacteriostatic or bactericidal, and are effective preservatives since they prevent bacterial growth in very low concentrations.

Complete Molecules. However, in the case of organic acids such as benzoic, salicylic, and acetic, we have also to consider the *undissociated molecule* since these acids dissociate very slightly. Their toxic effects are much greater than would be expected from equivalent concentrations of *p*H. For example, hydrochloric acid and sulfuric acid, although "strong" acids, are much less poisonous to most bacteria at a given *p*H than benzoic acid or acetic acid at the same *p*H; about 7.5 to 7.7 parts per million of the first two are required to produce the same toxic effect as 0.1 and 1.2 parts per million, respectively, of the last two.[7]

Magnetism. The subjection of microorganisms to magnetism has not been demonstrated to exert any important influence on them.

Electricity. The passage of an electrical current through a suspension of microorganisms probably has little effect by itself. If a current of great intensity be passed for a long time, however, electrolysis of some of the constituents of the medium will result, their nature and concentration depending on the voltage, and the composition of the medium and of the electrodes. Some of these products of electrolysis have deleterious effects. Heat, also, will be generated and, if sufficient, may kill the organisms.

Electrophoresis. The student familiar with colloids knows that any very minute particles, including microorganisms, when suspended in aqueous solutions, acquire an electrical charge on their surfaces.* Therefore, when an electrical current is passed through a suspension of such particles, those with a negative charge (anions) travel toward the positive electrode (anode), while those with a positive charge

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* The origin of this charge is not clear.

(cations) travel toward the negative electrode (cathode). This migration is termed *electrophoresis.*

The electrophoretic migration of the particles may be observed directly with a microscope focussed on a thin, hollow cell with electrodes at each end (Fig. 65) or by testing for their presence in the legs of a U-tube with electrodes at each leg, the particles being introduced at the middle of the U.[8]

This phenomenon has been used in the study of the electrical charge of various microorganisms. For example, it has been shown that a form of yellow fever virus with special affinity for nervous tissues has electrophoretic properties which differ markedly from those of the mosquito-borne form of the virus[9] (see page 815). Verwey[10] has shown that pathogenic micrococci migrate differently from micrococci of little pathogenicity (Fig. 66).

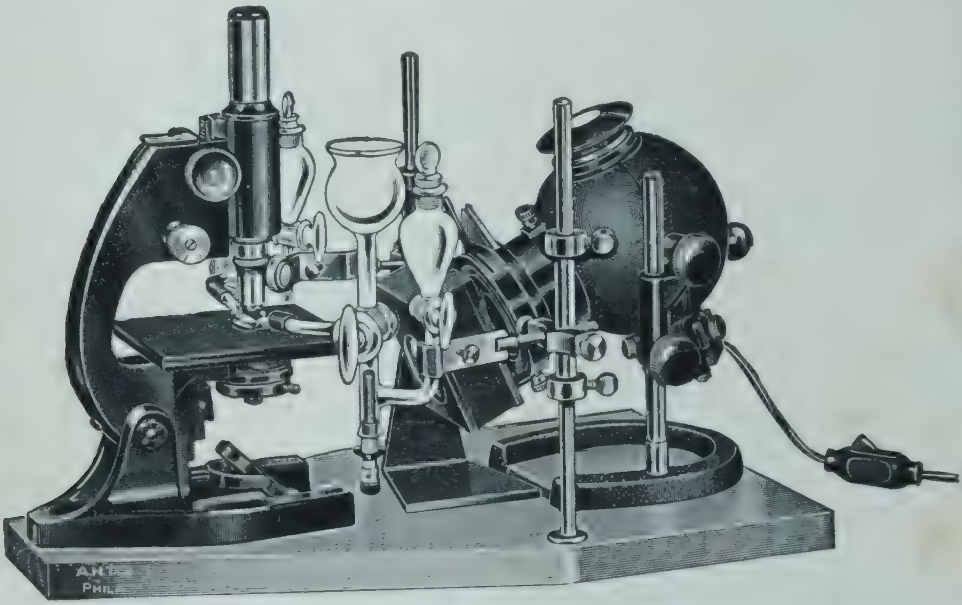

Fig. 65. One form of electrophoresis apparatus. The microscope is focused on a hollow glass slide containing the suspension of bacteria. The electrodes are seen below the stage of the microscope, on either side. The thistle tubes and other parts of the apparatus are used for flushing out the cell and changing the fluids around the electrodes.

Desiccation. While some bacteria are very sensitive to slow drying (see evaporation) many vegetative bacteria can withstand virtually complete desiccation for long periods, especially in the dark and cold, if the desiccating process be rapid. However, no growth occurs under these conditions.

Desiccation and Vacuum. Not only do many kinds of bacteria in a vegetative state withstand desiccation, but they may be frozen and subjected to the highest possible vacuums without harm. These facts are of the greatest interest from both a philosophical and practical standpoint.

In a state of virtually complete desiccation and in a high vacuum, there must be an unimaginably small vital activity. The metabolic processes must stop almost completely since these depend largely on diffusion, ionization and the colloidal state, all of which are dependent, in turn, on hydration. How, then, can bacteria exist desiccated in a vacuum? How can living things survive in the virtual absence of free water and free oxygen or, as it seems, in the entire absence of vital activity? There are no known answers to these questions. The condition of bacteria desiccated in a vacuum must be as near an approach to suspended animation as can be imagined.

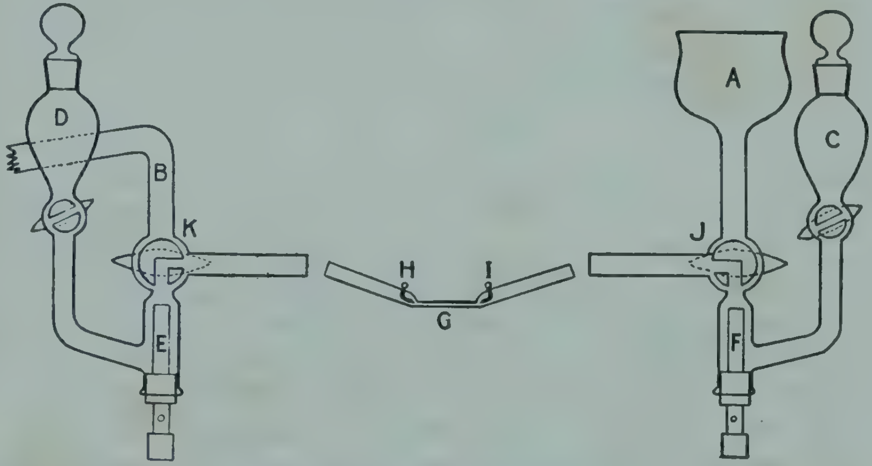

Fig. 66. The essential parts of an electrophoresis cell.

- *A.* Reservoir of the suspension of bacteria to be tested.
- *B.* Outlet tube, for passing off water used in flushing out the apparatus, etc.
- *C, D.* Reservoirs for saline solution bathing the electrodes and carrying the electric current from them to the suspension of bacteria which extends from *A* through *J, G* and *K.*
- *E, F.* Electrodes.
- *G.* The electrophoresis chamber proper within which actual microscopic observation of electrophoretic motion is made.
- *H, I.* Glass-insulated platinum electrodes for measurement of the potential gradient across the electrophoresis cell.
- *J, K.* Three-way stop cocks controlling flow of electrode fluid, bacterial suspension, or wash water.

If time for human beings be measured by successive events such as heart beats, thought train, action of enzymes, wars, etc., then when these activities or their effects cease to be *observed,* time ceases *for the individual* involved and begins again only when the individual consciousness again begins to pace exterior events or when vital activity produces the effects of age. A person deeply preoccupied by study, pain, play or worry, or who has been wholly unconscious, or in an abnormal mental state (delirium), in which consciousness of the course of events is temporarily lost, has a different idea of time from that of normal persons, or at least a very distorted one. An analogous situation exists for bacteria in a preservation vial; although they have no consciousness of passage of tim when the air and moisture are withdrawn from their environment, time may b imagined by us as ceasing to exist for them since their stream of vital activit and the process of aging apparently cease. Time (as measured by us for them begins again only when air and fluid are added. In the vacuum jar their chronological age may become great, as measured by human standards, but they appea to remain physiologically young and unchanged even for many years durin their sojourn in vacuity.

Preservation of Bacteria. From a practical standpoint, the surviva of bacteria when desiccated in a vacuum is of great importance. In the laboratory, in the past, in order to preserve pure cultures of non-sporeforming bacteria, it was necessary to transfer the organisms frequently to fresh culture medium, incubating and then maintaining them in a vegetative state in the refrigerator. This procedure involves danger of contamination of the cultures, and alterations in character due to changing environment. These, as well as loss of time, labor and cost of such methods can be eliminated where it is known that the species will withstand desiccation *in vacuo*. A simple method of drying and preserving bacteria *in vacuo* has been described.[11] Once preserved by this method, a large number of cultures may be stored in a pint fruit jar and many bacteria will survive for years. Species of pathogenic streptococci, for example, have survived unharmed for twenty or more years, while diphtheria bacilli survive for fifteen years and can probably survive much longer under these conditions. However, while many species of bacteria resist vacuum and drying this is not true of all. Many will survive in the frozen state at —76° C. that do not withstand desiccation.[11a] Certain bacteria (meningococci), hitherto supposed to be *very sensitive* to cold and drying, may be preserved desiccated in a vacuum for a year or more after first rapidly freezing them. Pure cultures of tubercle bacilli have been preserved in the same way.[11b]

Lyophile Process of Preservation. A method of preserving very unstable substances such as living microorganisms (see Chap. 46), enzymes extracted from the inside of living cells, bacterial toxins, etc., all of which are inactivated or destroyed on prolonged storage in a moist state or exposure to the air, depends on extreme cold and rapid evaporation. The materials to be preserved are suspended or dissolved in an aqueous fluid and distributed in small glass ampoules connected to a high-vacuum pump. The contents of the ampoules are frozen almost instantaneously by immersion in a bath of dry ice in methylcellosolve (—78° C.) and then dried from the frozen state by the application of a high-vacuum. The necks of the ampoules are sealed with a needle of flame before destroying the vacuum. The product is a highly *lyophilic* (water-loving) powder. A process employing a chemical drying agent, the Chryochem process, was described by

Flosdorf and Mudd;[12] a simplified apparatus of the same nature was devised by Taylor.[13]

Extreme Cold. Many species of bacteria are highly resistant to extremes of cold, even when in the vegetative state. In this respect they are almost unique among living things and it may be due to this that certain species of bacteria may have survived glacial epochs in the earth's history. Many species, even parasites like the typhoid organism, will survive for weeks frozen in ice. Turner et al. have maintained syphilis spirochetes and several viruses frozen in "dry ice" at —76° C. for years without loss of infectivity.[3] Some species of bacteria will grow, apparently unaffected, even after subjection to the temperatures of liquid hydrogen (—252° C.). Larger cells than bacteria are often entirely disrupted by the crystallization (freezing) of their fluids but some protozoa and metazoa have been held at —70° C. for long periods.[14]

Radiant Energy. The Electromagnetic Spectrum. As seen in figure 67, electromagnetic waves may be arranged according to length in a spectrum including the visible solar spectrum (red-violet), the latter being a small part of the whole system of electromagnetic waves. The physiological effects of these various radiations differ greatly, those in the shorter ranges being of most known importance. Very long waves are used in radio. The longest waves having known physiological effect are (1) the infra-red or "heating waves"; (2) the next shorter are the visible red rays, which affect the retina of the eye and also bacteriochlorophyll; (3) then come successively, yellow, blue and violet, which affect the retina and have photodynamic and possibly other biophysical actions; then (4) the ultraviolet rays, which are beyond the visible violet and have marked physiological and biochemical activities to be discussed. Still shorter waves of known properties are (5) x-rays ("soft" and "hard"); (6) gamma rays, and finally (7) the little understood cosmic waves.

Radium Emanations. Radioactive substances like radium give off three kinds of rays.

The so-called *alpha rays* of radioactive substances are in reality positively charged helium atoms given off at high velocity. The *beta rays* (or particles) are negatively charged particles, the exact nature of which is not certain. These also travel at high velocities. The *gamma waves* are really part of the electromagnetic spectrum in the very short wave-length range.

Wave Length and Penetration. In general, *below the visible* range the ability of these forms of radiant energy to penetrate through different substances varies inversely with their wave length. Thus, x-rays have much greater penetrating power than visible or ultraviolet light, as is well known to any one who has seen x-ray photographs.

If waves have great penetrating power they pass completely through living things exerting little effect. They are most effective when stopped or absorbed by a given substance as, for example, the chromosomes of a cell, or the nucleic acids of the cytoplasm (see section on microbial variation). As will be shown, the ultraviolet rays penetrate just enough to be stopped and absorbed by many biological materials and therefore are biologically a very effective form of radiant energy. The short, or "hard," x-rays have even greater ability to penetrate matter which stops all longer waves. They have relatively little effect on microorganisms. The still shorter gamma rays are like the very hard x-rays, with even greater penetrating power. Cosmic rays, shortest of all, may penetrate through many yards of earth and through thick concrete walls and lead plates.*

The alpha rays (or particles) have little penetrating ability, being stopped even by thick paper and thin films of other substances, as glass or aluminum. When they *do* penetrate, as into the surface layers of protoplasm or the cells of microorganisms, provided they are *absorbed* and do not pass completely through, they have intense ionizing powers since they liberate electrons of high kinetic energy. Cosmic rays have an even more intense effect. The beta rays (or particles) have greater penetrating power and are not much absorbed by such small objects as microorganisms, mostly traversing them completely, although prolonged exposures to beta radiations do have effects on some microorganisms. Gamma rays have little effect on microorganisms demonstrable by ordinary methods, and it is to be presumed that the still shorter and more penetrating rays also are completely unabsorbed by living organisms.[15] Ultraviolet and soft x-rays, therefore, are the most commonly used in physiological studies.

X-rays. Lea[15] and others[22] have shown that x-rays, especially soft x-rays have a bactericidal action. Non-lethal exposures induce profound changes in bacteria. Some of these changes involve morphology and other characters to a marked degree. Fission is interfered with and long filaments or chains or cells are often formed. Permanent, genetically stable variants occur under the influence of such radiations.

Ultraviolet Light. Ultraviolet light, like visible light, consists of a spectrum of "colors." These consist of different wave lengths of from about 4000 Angstrom units (A°) down to about 200 A° (see Fig. 67). Those between 2800 A° and 2300 A° are most effective biologically, the most effective length depending to some extent on the species of organism tested and the conditions of test.

From the microbiologist's viewpoint the most obvious, but not the most interesting, biological effect is death. Microorganisms of most

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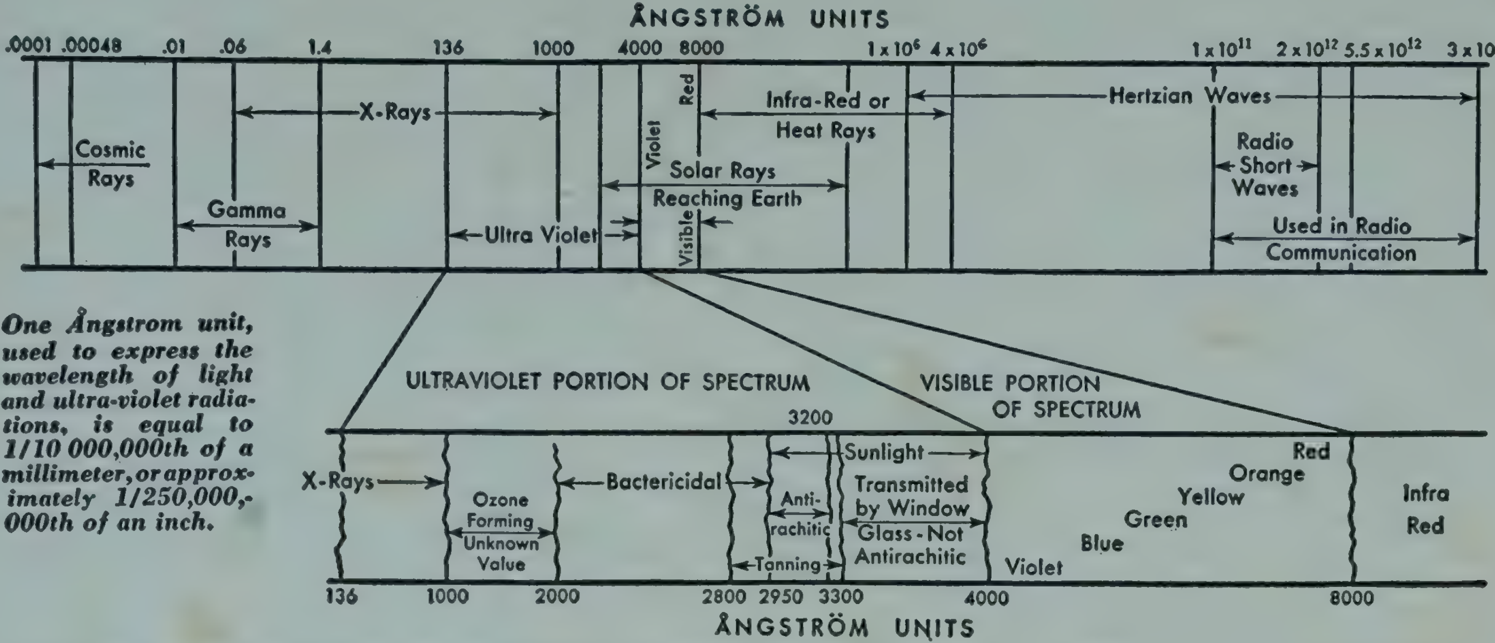
* Lead of proper thickness is definitely resistant to penetration by electromagnetic waves in general.


ÅNGSTRÖM UNITS
.0001
.00048
.01
.06
1.4
136
1000
4000
8000
1 x 10⁶
4 x 10⁶
1 x 10¹¹
2 x 10¹²
5.5 x 10¹²
3 x 10¹
Cosmic Rays
Gamma Rays
X-Rays
Ultra Violet
Violet
Red
Visible
Solar Rays Reaching Earth
Infra-Red or Heat Rays
Hertzian Waves
Radio Short Waves
Used in Radio Communication
ULTRAVIOLET PORTION OF SPECTRUM
VISIBLE PORTION OF SPECTRUM
3200
X-Rays
Ozone Forming
Unknown Value
Bactericidal
Anti-rachitic
Tanning
Sunlight
Transmitted by Window Glass - Not Antirachitic
Violet
Blue
Green
Yellow
Orange
Red
Infra Red
136
1000
2000
2800
2950
3300
4000
8000
ÅNGSTRÖM UNITS

One Ångstrom unit, used to express the wavelength of light and ultra-violet radiations, is equal to 1/10 000,000th of a millimeter, or approximately 1/250,000,000th of an inch.

Fig. 67. Spectrum charts. (*From The Westinghouse Sterilamp and the Rentschler-James Process of Sterilization, courtesy of the Westinghouse Electric & Manufacturing Company, Inc.*)

sorts, if irradiated with sufficiently intense ultraviolet (in the range of 2650–2750 A°) for longer than a certain time (the limits depending on the organism and the environment) die. This is valuable information and has been put to practical use in medical, public health, agricultural, commercial and research applications.[16, 17, 18, 19]

Radiating tubes giving off light of wave length about 2600 A° (2537 A° is commonly used in commercial ultraviolet lamps) have been put to use in preventing spoilage of meat, tobacco, bakers' goods and the like, due to molds and bacteria. Other uses of the radiations are the disinfection of glassware in restaurants, of "conditioned" air, the air in schools, hospitals and operating rooms, and in the control of insects (see section on disinfection). Use is made of ultraviolet light in the treatment of water in swimming pools and for drinking.

It is of interest to note that the effects of ultraviolet radiation are quite different from those of heat, since very thermostable spores are easily killed with ultraviolet light. Ultraviolet light also has inactivating or destructive effects on various proteins such as antigens and antibodies, and on enzymes, and toxins.

Genetic Effects. Among the most interesting and important effects of ultraviolet light are those resulting from less intense or prolonged radiation and involving changes in genetic structures such that mutations (new species?) are formed. The nature of the genetic changes induced, and the mechanisms involved, are discussed more fully in the chapter on Growth and Variation.

Darwin pointed out that fitness of new, genetically stable variants (mutants) to meet the environmental conditions imposed by Nature (natural selection) was the *sine qua non* of survival of the new type or species, and his conception of the origin of species was based on this idea. However, the *origin of the mutants*, the real, original, origin of species, escaped him and may have as its basis genetic changes induced by various influences such as radiations.

Action of Ultraviolet Light. The exact nature of the biological changes brought about by ultraviolet waves is not clear and probably differs in different substances and situations. That the action is not wholly dependent on oxidative changes, as is the action of sunlight, is clear from the fact that ultraviolet is as effective in a vacuum as in the presence of air. As pointed out by Lea,[15] "Some caution is required in the use of the method in which bacteria are irradiated while spread on the surface of nutrient agar since it has been found that large doses of radiation produce poisons in the nutrient medium which kill bacteria sown on it." The same source of possible error and many others, exist in studies with various forms of irradiation.

It has been demonstrated that the most affected compounds or structures in the cell are those which absorb the rays to the greatest extent. This has been shown in studies of the absorption, by gene structures and thymonucleic acid, of ultraviolet rays of single wave length (monochromatic light) (see chapter on Variation). The most destructive and lethal actions seem to be denaturation or coagulation of protein structures.

Photodynamic Action. A great deal has been said and written of the marked effects of ultraviolet and other forms of invisible radiant energy on living microorganisms but relatively little attention has been given to the biological effects of visible light rays (4,000 to 8,000 A°). These do not, in fact, appear to exert rapidly lethal or other striking influences per se, but under certain circumstances they produce death. For example, bacteria will often grow in contact with low concentrations of bacteriostatic dyes such as eosin, fuchsin, etc., provided no light reaches them. The combined effect of light and dye for only a few minutes usually kills the organisms. As shown by T'ung and Zia[20] and T'ung,[21] methylene blue, in the light of an ordinary electric lamp, rapidly kills certain gram-positive bacteria which withstand much higher concentrations of the dye in the dark. Gram-negative bacteria are not so affected. Other organisms, as viruses, and enzyme-like substances, proteins, etc., are also affected. The exact mechanism is not clear. Apparently waves of certain lengths are absorbed by certain parts of cells, or by certain substances, in the presence of dyes, and this brings about reactions in the proteins, etc., of a deleterious nature. It has been shown that oxygen is essential to photodynamic action, since the effects are not observed if the microorganisms are placed in a vacuum or in atmospheres devoid of oxygen. This is suggestive of the action of sunlight.

Sunlight. The disinfectant ("purifying") action of sunlight has been known for centuries and has been thought to be due mainly to the ultraviolet rays in solar light. That the effects of sunlight are not due exclusively to the ultraviolet waves is indicated by the fact that these effects of pure ultraviolet light are to a great extent independent of the presence of free oxygen. The phenomenon of photodynamic action also proves that the visible light waves may play some role in the action of sunlight. It will be seen in other sections of this book that even in the visible range different colored lights have different effects. Red light, for example, is most effective in activating bacteriochlorophyll (see page 453).

The heating and drying effects of sunlight are also important in its bactericidal effect.

Hydrostatic Pressures. If living microorganisms suspended in aqueous fluid are placed in a strong steel cylinder and a piston is pressed down upon the suspension with great force, the organisms may or may not be affected, depending on the species of organism. Many microorganisms will withstand pressures of 10,000 pounds per square inch without obvious injury. In the range above 90,000 pounds, however, death of many species occurs within one hour. Spores of various species may withstand pressures of 300,000 pounds per square inch. Such pressures inactivate some enzymes.

The changes brought about by high pressures include increases in rate of some chemical reactions, diminution in volume of water, increases in viscosity of many fluids, increased electrolytic dissociation, and others. Probably all of these changes are involved in the biological effects of high pressures, but exact details are obscure.[23, 24, 25]

As pointed out by ZoBell, "In interpreting experiments on the effect of hydrostatic pressure on bacteria, cognizance must be taken of the fact that the results are influenced by the temperature and composition of the medium. The rates of compression and release of the pressure are also important factors."

Marine bacteria are resistant to pressures at the bottom of the sea, which do not exceed 15,000 lbs. per square inch.[26] However, according to Johnson and Lewin, 1,000 lbs. per square inch retards *E. coli* at suboptimal temperatures and 5,000 lbs. kills. Growth is accelerated at higher temperatures. At 8,000 lbs. at 48–50° C. the killing effect is lessened.[27]

While bacteria are fairly resistant to pressures of fluid against them, they are easily crushed by the impingement upon them of solid particles like steel or glass balls, or in freezing liquids, by ice crystals. Shaking in a round vessel with steel balls (ballmill) is a method used for the mechanical destruction of these organisms. They shatter especially readily if made brittle by freezing and this method is used for certain chemical investigations of bacteria. Shaking for several hours of a bacterial suspension mixed with fine beads of glass has been shown by Curran and others to accomplish a complete disruption of the cells.[28]

Osmotic Pressure. The student will doubtless have learned in his courses in biology and physics that if a semipermeable membrane like fish-bladder or egg shell membrane separates two solutions, one having a high salt or sugar concentration and the other a low one, water will tend to pass (diffuse) through the membrane toward the solution of higher concentration till the concentrations are in a state of osmotic equilibrium on both sides. The solution of higher concentration is said to have a higher *osmotic pressure* than the other. If living cells are immersed in fluids having extremely high or low osmotic pressures, the normal selective permeability of the cell wall may be overwhelmed, for a time at least.

Well-known examples of the effects of unfavorable osmotic pressures are found in the plasmolysis and plasmoptysis seen in cells of certain plants when they are immersed in solutions having too high (hypertonic) or too low (hypotonic) a salt concentration. Likewise, if we immerse certain bacteria, especially delicate parasitic forms, in a solution of very high osmotic pressure, water is drawn from them and they may be injured or die before equilibrium can be established. If they are immersed in distilled water the reverse process may occur with deleterious effects. It is partly for this reason that the osmotic pressure of fluids used for the cultivation of bacteria is adjusted by the addition of measured quantities of salt or other substances. The main purpose is to prevent hemolysis of blood which may be added to the medium. Most bacteria are not very sensitive to variations in salt concentrations below about 1 per cent. Concentrations much above this may adversely affect some of the more sensitive strains. Much depends on the previous environment of the organisms. ZoBell and others have shown that marine bacteria, adapted to the salinity of ocean water (about 3.5 per cent) are quite sensitive to lower or higher salinities and will not grow if the salinity is less than about 2 per cent or over about 15 per cent. On the other hand, if media intended for colony formation by bacteria adapted to life in fresh water lakes be given a salinity as high as 1 per cent, no colonies will be formed by certain aquatic species. Bacteria from either source, however, may be adapted to the environment of the other.

As shown by ZoBell there are bacteria which have become adapted to the high salinity (around 29 per cent) of various salt waters such as the Dead Sea and the Great Salt Lake of Utah. These organisms will not grow in lake water diluted to a salinity of less than 13 per cent. Such organisms are spoken of as *halophilic*. On the other hand, the growth of soil, sewage or mouth bacteria is inhibited by the lake water and ordinary marine bacteria are killed by a few minutes' exposure to it. There are halophilic bacteria which grow in commercial pickling brines. Some are a cause of spoilage of various commodities preserved with salt, such as fish, meat, and hides. Concentrations of salt around 25 and 30 per cent are used in these brines.[29, 30]

Effects of Evaporation. When a solution containing salt or other soluble compounds is exposed to the air so that evaporation occurs *slowly,* the water, departing, leaves behind a more and more concentrated solution, the osmotic pressure becoming greater and greater. Bacteria suspended in such a fluid are unfavorably influenced by the increasing osmotic tension. In the cultivation of bacteria, therefore, evaporation of moisture from the medium is to be avoided. This may be accomplished by maintaining a very humid atmosphere in the incubator.

Alterations in Permeability. Involution Forms. Even though the osmotic pressure of a given culture medium may be appropriate, there often appear in it substances that alter the permeability of the cell wall in some way so that the cells become swollen and distorted. Such alterative substances may be the waste products (acids, alcohol, etc.) of bacterial cells which have grown in the culture. The cell wall may also become altered through aging so that it fails to function properly, very likely permitting the passage outward of important cell constituents and the passage inward of water or of deleterious compounds and probably, through localized weakenings, causing the swellings, knoblike protrusions and other irregularities often observed in old cultures.

These swollen and distorted forms of bacteria have been observed by bacteriologists for many years and generally are spoken of as "involution forms." In certain species (*e.g., V. comma*) such distortions may be brought about to a very striking extent by cultivation of the organisms on media containing high concentrations of sodium chloride (Fig. 68). Many modern students of the subject, however, attach importance to some of the irregular forms of bacteria as representing stages in life cycles and complicated reproductive processes. The whole question is an open one and those who have time and inclination to investigate it will find much of interest and value.

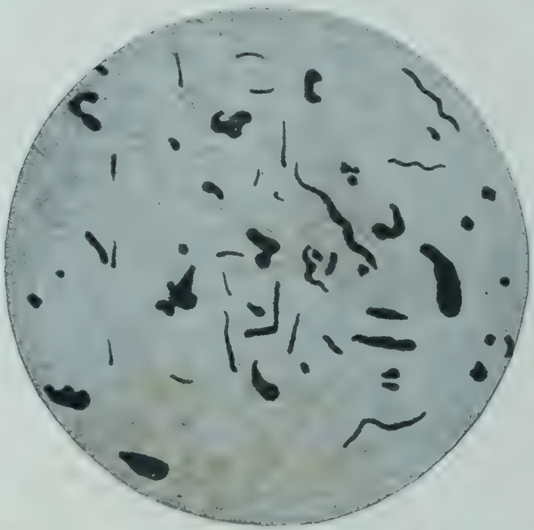


Fig. 68. Involution forms of *V. comma* (cholera vibrio) when cultivated upon agar containing excessive amounts of salt. (Compare with Fig. 265.)

Surface Forces. Because of their very minute size bacterial cells themselves, as well as their constituent colloidal protoplasm particles, have many of the properties of large colloidal particles (see page 269). For example, in aqueous suspensions near pH 7 most colloidal particles, as well as bacteria, possess negative electrical charges.

The relationships of the volume of microorganisms, like bacteria, to their surface area are also like those of colloids. For example, a very small mass or volume of bacteria, say 1 gram, contains thousands of billions of cells, each of which presents to the environment a chemically and physically reactive surface. The sum total of all of the cell surfaces in such a mass reaches magnitudes in the range of 10,000 square centimeters or the area of a small room. It is, therefore, readily understood that, since protoplasm, and microorganisms in general (especially bacteria), have a high surface: volume ratio, forces which act at surfaces will profoundly affect physico-chemical processes which occur on the exterior surfaces of bacteria and similar microorganisms, as well as among intracellular protoplasmic colloidal particles. Surface forces, in fact, are of the most profound importance in all cell life and obviously we should know something about them. One of the most important of all is called *surface tension.*

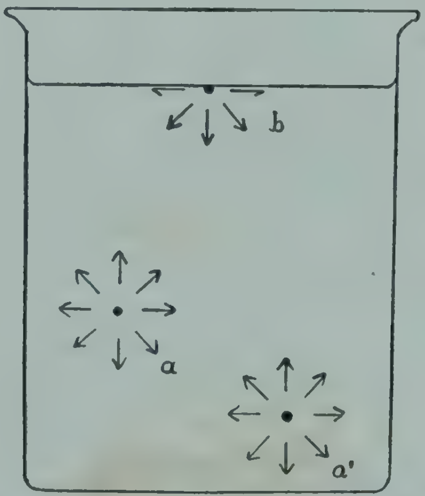


Fig. 69. Diagram showing how surface tension acts. The molecules *a* and *a'* are attracted equally from all directions and are in a state of equilibrium. Molecules at the surface, like *b*, are under a greater tension from below than above and the entire surface therefore tends to pull inward much as though the surface film were a rubber membrane.

Surface Tension. It will be remembered that there exists an attraction or cohesive force between molecules. The attraction exists in all matter and is evidenced, in fluids, by the action of their surfaces. It may be illustrated as follows:

If we imagine a single molecule in a beaker of pure water (Fig. 69, *a*), a few centimeters below the surface, it is obvious that the attraction of the surrounding molecules is equal on all sides. When this molecule is at the surface (Fig. 69, *b*), the attraction of the large number of liquid molecules in the water below is much greater than that of the few in the vapor above the surface of the liquid. The same is true of the molecules at surfaces in contact with the glass walls of the vessel or any particle suspended in the water; *i.e.,* the molecular pull is mainly from only one direction. It is easy to see that the pull upon all the molecules at the *surfaces* of the liquid is greatest from below, or within the bulk of the fluid, and that the surface of the fluid mass will therefore tend to be drawn in. It is this force which enables us to fill a beaker to a level several millimeters above the actual limits of the glass walls. The intermolecular pull results in *surface tension.* Under its influence the fluid surface acts, in many respects, like an elastic membrane.

Adsorption. Of the many phenomena which result from the action of surface tension, probably the most important and best understood is *adsorption.* Explanations of adsorption and surface tension are based on the *law of entropy.* This law requires that any change which can reduce the amount of potential energy in the universe shall tend to occur to a maximum degree. Surface tension is a form of potential energy. Suppose a substance (*e.g.,* soap) which reduces surface tension is added to the water in the beaker shown in Figure 69. It must, according to the law of entropy, tend to carry the reduction of surface tension as far as possible. This may be accomplished in several ways. For example, the actual extent of the surface may be reduced by contraction or shrinkage in volume. Should this be impossible, as in this case, the surface tension reducent can lower the amount of potential energy to the greatest extent only by accumulating at the surfaces of the fluid. This accumulation is known as *adsorption.* Deposition of the surface tension reducent may then occur on the air-fluid surface, at the surface in contact with the glass walls of the containing vessel and on the surface of any particles, such as bacteria, suspended in the fluid.

It has been shown that substances adsorbed upon a surface are often greatly *concentrated* and that, in many cases, their *solubilities increase.* It is probable that some gases are actually liquefied when adsorbed. It is clear that such condensations and increases in solubility must, from the *law of mass action,* greatly facilitate chemical reactions, since the law of mass action states that the greater the concentration of the reacting substances the more rapid the reaction between them. Catalytic agents, discussed in the chapter on enzymes (see page 264) depend upon this law, which governs molecular contact.[31]

As will be seen later, nearly all of the chemical activities, both favorable and unfavorable, occurring in and on living cells, are influenced by adsorption and surface tension and the phenomena should be well understood at this point (see also section on disinfectants).[32, 33]

Wetness. An important result of the power of substances to lower or weaken surface tension lies in the fact that biologically important fluids of all sorts—cell fluids, disinfectants, culture media, etc., having low surface tension spread better, *i.e.,* are actually *wetter,* than other solutions. This brings them into more intimate and effective contact with surfaces such as colloidal particles, cell membranes, etc. Mercury, for example, although a fluid, has a very high surface tension and therefore, as everyone who has held mercury in the palm of the hand knows from experience, possesses the property of wetness only in a very slight degree. Water has a moderately high surface tension, and a moderate degree of wetness, while alcohol has a much lower surface tension. Alcohol, therefore, wets or spreads when in contact with surfaces much better than does water and thus comes into more intimate contact with objects which it touches. The importance of low surface tension and wetness of many disinfectants, culture media, etc., will be mentioned later on.

Sound. Bacteria, because of their small mass and cell wall, are not readily disrupted by vibration. Sound vibrations within ordinary ranges from very low up to very high rapidities apparently have little effect as a rule. The "high" limit of audible tone has a vibration rate of around 30,000 per second, the lowest audible tone has a vibration rate of around 30 per second. However, a sonic vibrator consisting of a nickel tube, motivated by means of an electromagnetic field, has been devised which emits sound waves with a frequency of 9,300 vibrations per second. This particular tone is lethal for some species of bacteria, as well as one type of bacteriophage.[34] *Supersonic vibrations* are so rapid that even bodies having the small inertia of bacterial cells are shaken asunder when subjected to their influence.

The sonic or supersonic waves must be of such pitch that the cells to be disrupted vibrate in "sympathy" or harmony with them. The vibrations must also be of considerable intensity.

These methods of shattering bacteria are of value in experiments where it is desired to kill and disrupt the organisms without producing such profound chemical and physical changes as result from heating, chemical destruction, exposure to sunlight, or age. Delicate poisons, proteins, carbohydrates or enzymes inside the cells have been liberated in an unaltered state by supersonic vibrations and may thus be studied in an unchanged condition.[35] Particles as small as viruses may be very resistant to vibrations.[36, 37]

Natural Environments. Under conditions in nature many of the factors affecting the environment of microorganisms are determined by the growth of other organisms; for example, shortages of food or oxygen, unfavorable pH, antibiotic substances, etc. If we eliminate these naturally occurring factors, as is generally done in pure culture studies, we obtain a curiously unbalanced knowledge of microorganisms.

Consider, for example, some minute recess a few inches below the surface of a soil rich in organic matter and moisture. Here we find strict aerobes and anaerobes growing, decomposing cellulose and proteins to simple substances available to many other soil microorganisms. These and other organisms produce compounds of an endless variety, all available to the soil flora and fauna. The growth of the anaerobes may be favored by the utilization of excess oxygen, or catalase production, by the aerobic bacteria. Here also the fertility of the soil may be increased because autotrophs grow, forming nitrites from ammonia produced from proteins by the proteolytic heterotrophs; and nitrobacter producing nitrates from the nitrites. At the same time, all of these bacteria may be held in check and perhaps killed by the poisonous substances poured forth by certain of the *Actinomyces,* molds and *Pseudomonas* group, spore-forming organisms alone outliving all others. The constant necessity for adjustment to, or the selective effect of, changing temperatures and changing osmotic pressures, as well as changing composition of suspending fluid due to fluctuation of water—conditions which are fairly constant in most artificial media—also affect microorganisms.

It is plain that environmental vicissitudes and interactions between various forms of life are the usual experiences of free-living microorganisms, as contrasted with growth in isolated tubes and completely segregated artificial communities (pure cultures) under the most absolutely uniform and favorable conditions possible in the laboratory.

Even those strict parasites which never survive in nature outside the body of their host seldom find the site of their entrance into, or growth upon or in, the body devoid of other bacteria. One need only think of the mixtures of organisms in the oral, nasal and intestinal cavities. The science of immunology has taught us something about reactions between pathogenic bacteria and their animal hosts, and we know that each produces alterations in the other. Our knowledge of the nature and effect of the saprophytic or potentially pathogenic bacteria often present at sites of infection, upon invading organisms is, however, relatively slight.

The point is that laboratory studies generally fall far short of being exact investigations of microorganisms because they do not reproduce the natural environments of the organisms.

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Chapter 7

THE CULTIVATION OF MICROORGANISMS

In the early days microorganisms were found chiefly associated with organic matter. They were first seen by Leeuwenhoek in such material. The early experiments on spontaneous generation of life were made with hay infusions, meat, dung, etc. Pasteur called attention to their presence in fermenting wine and beer, while Koch studied them in blood and pus and other body fluids. As the science of microbiology grew and laboratory methods were improved, certain organic materials of moderately constant composition came into common use for cultivating microorganisms: milk, infusions of meat prepared in certain prescribed ways, blood, serum, meat partly digested by pepsin (peptone), vegetable infusion, slices of potato and other vegetables, and so on. These are referred to as culture media. These substances long held, and still hold, favored places in the supply room of the microbiologist.

As knowledge grew certain improvements were made: *p*H was carefully controlled; buffers were used; conditions of temperature, salinity, aeration, etc., were adjusted to the requirements of various organisms as these became known.

As biochemical data accumulated, still more precise methods were developed. It was found possible in some instances to abandon the use of organic matter of wholly or partly unknown composition, such as meat infusion, peptone, serum, urine, etc., and to formulate quantitative solutions of chemically pure substances, like salts, amino acid, vitamins, etc., which serve very well for many microorganisms. Such mixtures are called synthetic culture media. This phase of microbiology is still under very active investigation. For many routine purposes, however, the older organic or empirical media are still widely used.

The selection of a medium appropriate to the cultivation of a species of microorganisms must depend, to some extent, on a knowledge of the food and other requirements of that species. It has been found that all microorganisms may be divided among two large, rather vaguely differentiated and overlapping groups, the *autotrophic* and *heterotrophic*. The latter includes the *saprophytic* and *parasitic* species.

Autotrophic, Saprophytic and Parasitic Bacteria. The first forms of life may be imagined to have had for their nutrition only simple, inorganic materials, which may be all that were available in the primordial oceans and atmosphere. Among these, probably, were carbon dioxide, hydrogen, oxygen, sulfur, iron, water, and simple, inorganic salts, including phosphorus and ammonium salts, the last named serving as a source of nitrogen. Possibly simple organic substances also existed. From these our primitive ancestors must have built up their complex chemical structure (protein, etc.), obtaining their energy from oxidative processes going on inside them. Organisms having this type of metabolism still exist, widely distributed in the soil and water, and are called *autotrophic* (*auto* = self; *trophic* = nourishing) bacteria. These are characterized especially by the fact that they utilize inorganic sources of carbon: CO_2 or carbonates. Some may also use organic carbon and it is here that we find one of the overlappings mentioned above. Many, however, live on wholly inorganic media. They characteristically do not obtain their energy from their carbon sources. This is an important point and will be mentioned later repeatedly.

It seems likely that, later, or, according to some views, at the same time, forms appeared which utilized complex organic food materials such as the dead bodies or waste products of their fellows and that these forms initiated and still aid in, the decay processes so essential to soil fertility and to the development and continuance of "higher" life. Such types are spoken of as *saprophytic bacteria* (*sapro* = decay; *phyte* = plant). Most of the bacteria known today are of this type, and decomposing organic matters, such as feces, putrefying animal bodies, plant and animal matter in the soil and hay infusions contain large numbers of them.

Probably still later there appeared, either through progressive or regressive stages or both, bacteria which could live not only upon dead and waste organic matter, but which could also metabolize *living* plant and animal tissues. They caused disturbances of the delicate chemical and physical equilibria of the creatures on which they lived. This was disease and often resulted in the death of the invaded creatures. Such organisms are well known today and are called *parasitic* or *pathogenic bacteria* (*patho* = disease; *genic* = producing).

The saprophytic and parasitic or pathogenic species are grouped together as *heterotrophs,* which are characterized by requiring organic sources of carbon such as glucose or amino acids. However, some, perhaps all, can also utilize CO_2. Some requiring organic carbon may be like autotrophs in all other respects: able to live on inorganic sources of all other necessary elements. Many of them, however, require also organic nitrogen sources, and other complex organic compounds such as vitamins. In contrast to the autotrophs, heterotrophs obtain their *energy* from organic carbon compounds, especially carbohydrates. It is important to remember this. They may also require CO_2 for the *synthesis* of organic compounds.

Both autotrophic and heterotrophic bacteria must have whatever food they use in a completely dissolved or digestible condition.

CULTIVATION OF HETEROTROPHIC BACTERIA

Like all living things, bacteria must have water and sources of important elements, especially, C, H, O, N, S, P, Ca, Na, K, and others. These must be in an assimilable form. For many autotrophic species, the simple elements or inorganic compounds of them will serve. For heterotrophs, many of them, *especially carbon,* must be provided in the form of organic matter.

Sources of Organic Matter. *Animal tissues.* It has been found that extracts of flesh furnish all of the nutrient requirements of many heterotrophic microorganisms.

Two sample formulae given below differ in respect to the type of meat extract used. One ("extract broth") utilizes concentrated extracts like Difco or Liebig's *"Extract of beef."* The other, a much "richer" medium, which supports the growth of a much wider variety of microorganisms, especially pathogens, requires that freshly ground meat be soaked or *infused* in water for two to twenty-four hours, and the juices squeezed out and used. Broth made with fresh infusions of the latter kind is called "infusion broth." The virtue of fresh meat infusion probably lies in the fact that a greater variety of organic substances, including certain vitamins or "growth factors" (see chapters on metabolism), is present, along with whole proteins, carbohydrates and other organic complexes, many of them in a colloidal state.

It may be pointed out that meat is not the only useful source of organic matter. Extracts of vegetables of various kinds are often used. Some workers use the flesh or juices of shellfish, while those interested in the bacteria of milk sometimes use whey, skim milk, etc. Eggs are often used also, especially for tubercle and diphtheria bacilli. An infusion of partly digested soybeans has recently been shown by Brewer to be as good as meat infusion for many purposes.

Some media are made by adding bits of kidney, spleen, or other tissues freshly removed from dead animals under aseptic precautions to tubes of broth. The incorporation of small amounts of some solid or jelly-like substance such as ground meat or even sand is often advantageous as many microorganisms seem to grow best in the crevices of, or in contact with, the surface of such matter, forming little nests or *niduses* there. It may be that oxygen or food substances concentrate at such points by adsorption. In very dilute media, growth may occur at such surfaces and not elsewhere.[1, 2] The wide practice of clarifying fluid media by filtering out all insoluble precipitate, "for appearance's sake," may therefore be unnecessary and even detrimental. For some purposes, however, water-clear media are necessary. (See Fig. 70.)

Peptone. Peptone consists of a mixture of the products of partial protein digestion and includes many amino acids, polypeptides and proteoses, as well as other substances which result from the incomplete digestion of protein. The exact composition of peptone is not known, and is probably highly variable; but it is particularly useful for microorganisms which cannot attack whole, undigested proteins like egg, or blood, and which cannot utilize simple elements and inorganic materials.

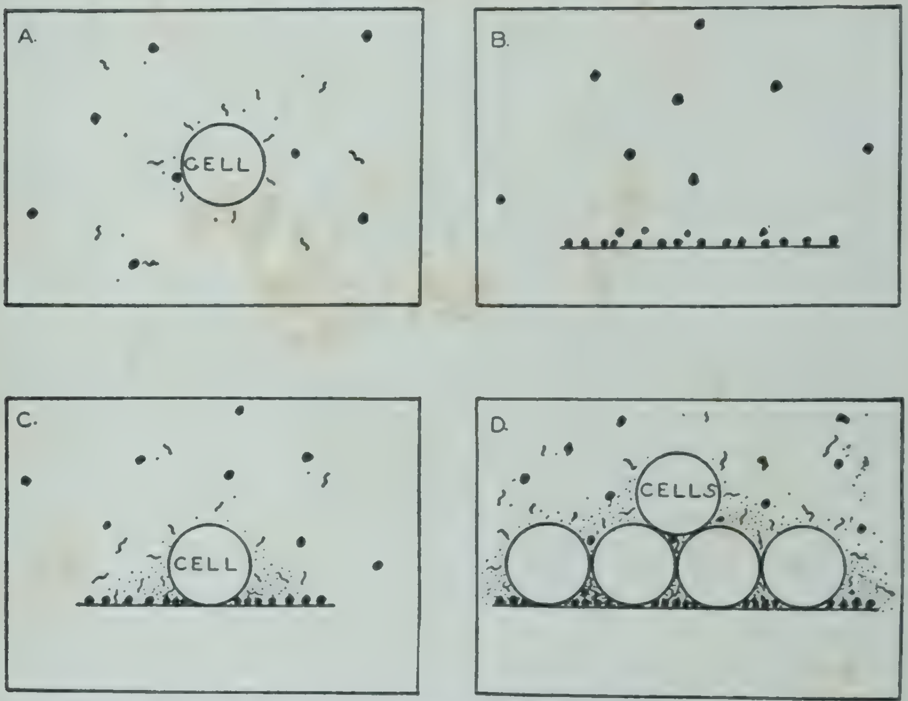

Fig. 70. *A*, A free-floating bacterial cell surrounded by a few suspended particles of food (dark circles) which must by hydrolyzed by the exoenzyme (helicoidal lines) before the resulting hydrolysates (dots) can be ingested and assimilated. *B*, Particles of food concentrated in a monomolecular layer on a solid surface. *C*, Food particles are more available to the cell on the solid surface where the interstices at the tangent of the bacterial cell and the solid surface retard the diffusion of exoenzymes and hydrolysates away from the cell. *D*, Multiple cells form additional interstitial spaces. (ZoBell, Claude E., in J. Bact., vol. 46.)

Along with the organic carbon and nitrogen in peptone and meat extracts there are associated, of course, oxygen, hydrogen, sulfur and phosphorus, in organic forms which are available to microorganisms, as well as certain vitamin-like factors. Peptone, therefore, is a very useful source of organic matter and is used in both of the formulae to be described.

I. *Meat Extract Broth:*

Beef extract	3 gm.
NaCl	5 gm.
Peptone	10 gm.
Water (distilled)	1000 ml.

The various ingredients are dissolved in the water as it is heated. Boiling is not necessary at this point. As soon as they are dissolved the *p*H is adjusted by the method described above and the medium, after boiling, filtration and sterilization is ready for use.

II. *Meat Infusion Broth.* The formula using infusion of fresh meat is comparable to the above in every way except that, in place of meat extract, 500 grams of freshly ground, lean beef, pork, veal or other meat are soaked or infused in a liter of distilled water in the refrigerator for two to twenty-four hours. The mixture is then heated to 80° C. and the juice filtered off through gauze or squeezed out by means of a meat press. Sediment is allowed to settle out in tall cylinders. During this time fat also collects on top and is removed. The volume at this point is usually a little over a liter but, if less, can be made up by the addition of water. Not more than about 100 ml. of water should be thus added. Peptone and salt are added as for extract broth and the medium is prepared as described before. Instead of filtration through paper, however, such material is usually passed through a thin towel or a layer of absorbent cotton enfolded in gauze.

Filtration is said to remove certain nutrient substances and should be omitted except when perfectly clear broth is necessary.

These two media form the basis of many cultural procedures and will be referred to repeatedly later.

The *p*H of Culture Media. The greatest care in details of preparation will, however, be of little avail if strict attention is not given to the acidity or alkalinity of the medium. This factor is of great importance and, while easily controlled, may constitute the difference between success and failure in cultural work. The reaction in terms of *p*H must therefore be carefully measured. This is most easily done by means of a special potentiometer or "*p*H meter." It may also be done by means of certain dyes which take a definite color at given acidities or alkalinities. The details have been given in Chapter 6.

Difficulties with Mixed Cultures. The early microbiologists had to contend with the difficulty that as soon as anyone attempted to cultivate organisms or handle them with instruments, they became mixed with a variety of microorganisms from the instruments, from dust, or from some other source. It was often impossible to distinguish one kind of microorganism from another in such contaminated cultures since many entirely different kinds look exactly alike when viewed with the microscope; even when stained by Gram's method. Pasteur, Koch and many others had the same difficulty. No one could be sure that the chemical or physiological properties he assigned to a given culture were really due to one kind of microorganism alone and would remain constant. It was like trying to determine the properties of a salt or element in solutions which contained other salts and elements. It was always a question whether the observed reactions were due to a single kind of organism or to a combination of them growing together in a culture vessel. Such mixed cultures would change their properties as soon as one or the other of the different kinds died off or gained the ascendancy.

Origin of Pure Culture Technic. Koch had observed the growth of mold and of different sorts of bacteria in isolated masses (called *colonies*) of various colors on slices of decaying potato in his wife's kitchen. One day, being in an investigative mood, he thrust a slender, sterile, platinum wire into one of the gray bacterial masses and put a bit of it in a little water between two thin slips of glass, under his microscope. Here he saw a great number of bacilli swimming about. Each one of these bacteria looked exactly like the thousands of others in this particular colony. Then Koch examined the bacteria from a yellow colony on the potato, and then those of a red one and of a violet one. The organisms from one colony were all round; from another they all had the appearance of tiny cylinders; from a third they looked like minute, living, spiral springs—but *all the microorganisms in any one colony were always exactly the same*. It was obvious that, by cultivating microorganisms on solid food, he could obtain isolated colonies of any kind.

The Use of Gelatin. Extending this principle it was but a step to the use of gelatin to prepare a transparent, solid, sticky, nutrient surface on flat pieces of glass. In addition, various nutrient infusions and test substances could be added to the gelatin before it was allowed to "set." Here was a very important advance—a revolutionary advance—one that has been the basis of all our present-day bacteriology. Thenceforth the study and discovery of bacteria in *pure culture* and by relatively exact methods became a matter largely of patience and hard work.

In summer, however, and when held in body-temperature incubators, the gelatin melted and this spoiled everything. It was then no better than the old fluid medium. Being a protein it was often digested and fluidified by the metabolic processes of the bacteria. Furthermore, when liquid, it spilled off the flat pieces of glass on which it had been allowed to "set," and besides that, particles of dust settled on it with various microorganisms which contaminated it, obscuring and confusing the results as badly as ever. However, the method was improved, as described in the next paragraph.

First Use of Agar-Agar. Many students flocked to Koch's laboratory from all over the world to learn his methods. One of these was W. Hesse. To the wife of this man the science of microbiology is indebted for suggesting, as a substitute for gelatin, the jelly-like substance derived from seaweed, *agar-agar*. Agar is transparent, colorless, bacteriologically inert, *i.e.,* is not digested or liquefied by most bacteria, melts only at boiling temperature and, once melted, does not set again till about body temperature. Agar has not been improved upon as a solidifying agent for culture media and is in general use for this purpose today.

Origin of the Petri Plate. In order to prevent contamination of the pure cultures by dust, another student in Koch's laboratory, R. J. Petri, suggested the simple expedient of pouring the melted, nutrient agar into flat, shallow dishes, and immediately covering them with a glass cover (Fig. 71). This permitted prolonged examination of the cultures but excluded dust. Such dishes are widely used today and are called Petri plates or Petri dishes (Fig. 72).

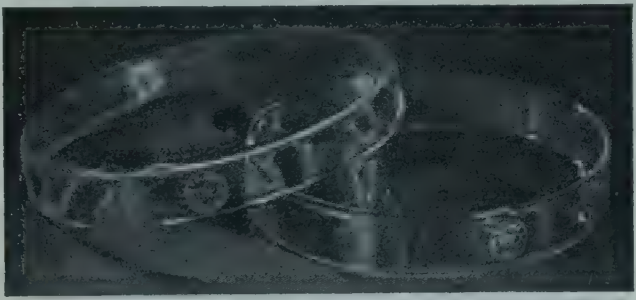

Fig. 71. Modern type of Petri dish.

The preparation and study of pure cultures then proceeded at a great rate all over the world.

Preparation of Solid Media. Today, procedures almost exactly like those devised by Koch and his associates are in general use in all bacteriological laboratories. The preparation of solid media is comparatively simple. To either of the fluid media prepared as indicated above, gelatin may be added in 10 per cent concentration while the fluid is still hot. Since, as Koch found, this liquefies so readily, agar is usually substituted following Frau Hesse's suggestion. Agar is used in 1.5 to 2 per cent concentration. These solidifying agents are added before the adjustment of pH.

When using agar, half of the water is withheld from either formula and the agar is melted in this by heating in a steam chamber or over a double boiler. The melted agar is then added to the hot extract-peptone solution or meat infusion.

Other solid media may be prepared by mixing the desired ingredients with eggs or serum, distributing in the desired vessels (usually test tubes), and then heating the vessels till the serum or egg coagulates. A smooth, firm surface results if the heating is carefully done.

Silica Gels. Silica can be made into jelly-like material with surfaces sufficiently solid to be inoculated like agar or gelatin. The advantages of silica as a solidifying agent are that, supplemented with an appropriate mixture of nutrient minerals, it will support the growth of isolated colonies of a number of species of autotrophic soil microorganisms which are injured by organic substances, such as the agar, eggs, serum or gelatin ordinarily used as solidifying agents.

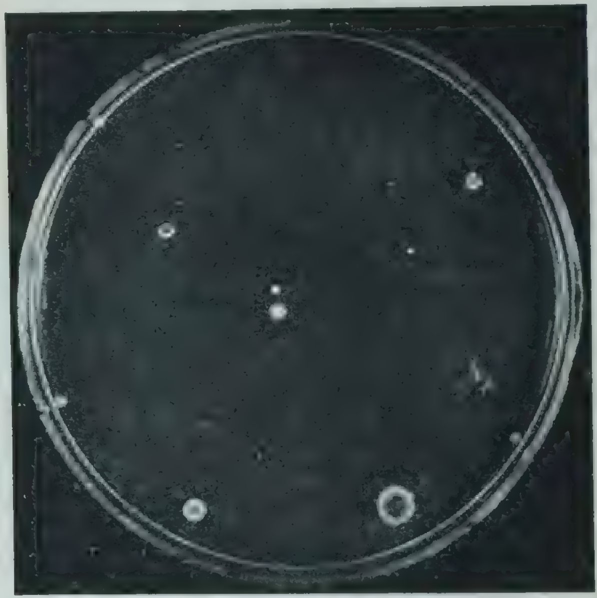

Fig. 72. Petri dish with isolated colonies of bacteria from the air (¾ actual size).

Various methods for the preparation of solid inorganic media by means of silica gels have been described since 1890. A method described by Moore[3] includes various nutrient substances which may be altered at will within certain limits.

Other formulae for silica acid jellies for bacteriological use were introduced by Hanks and Weintraub,[4] Sterges,[5] and by Ingelman and Laurell.[6] A discussion of their uses in soil microbiology has been given by Waksman.[7]

Special Media. To any of the broths (or nutrient gelatin or agar or silica gel when in a fluid state) various test and experimental or nutrient substances may be added. Certain carbohydrates may be included to test the fermentative powers of various organisms. Organic esters, blood, glucosides, and many other compounds are put into the medium for a great variety of purposes. The medium is then referred to by the name of the special substance; for instance, "blood-infusion-broth"; "serum-dextrose-extract-agar"; "starch-carrot-whey-agar" and so on.

Media for Fermentation Tests. Under this term are included all media by which the fermenting or hydrolyzing powers of organisms are tested with respect to carbohydrates, organic esters, and also certain glucosides and alcohols. These substances are added to suitable media usually in a concentration of 1 per cent before final autoclaving. Substances which are decomposed by heating may be sterilized by passing their aqueous solutions (usually 10 per cent) through porcelain, asbestos, or kieselguhr filters, and may then be added under sterile precautions to the medium before use. An indicator, which will change color when and if acid or alkali is produced from the test substance by fermentation, is added before sterilizing the medium, or after incubating the culture. Bromcresol purple is commonly used. Neither phenol red nor methyl red should be added before incubation as they may prevent growth of some bacteria. Inner inverted vials are often used to trap any gases formed (Fig. 73).

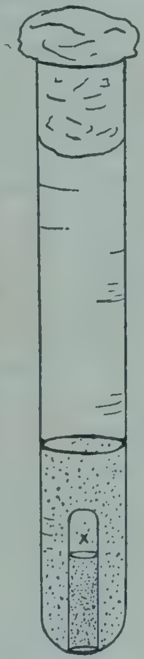

Fig. 73. Culture tube showing inverted inner vial for collection of gas resulting from fermentation. Some gas has collected at *X*.

Blood and Serum Media. Blood or serum for media must be obtained under sterile precautions. In small amounts (1 to 50 ml.) blood is conveniently obtained from rabbits, either by incising the marginal veins of the washed, shaved, and disinfected ear or, more simply, by tapping the heart with a needle. The blood may be kept fluid (unless serum is desired) either by defibrinating (shaking for at least seven minutes with glass beads) or by using 0.2 per cent oxalate or 1 per cent citrate to prevent clotting. (Use 2.5 ml. of 20 per cent sterile aqueous sodium citrate in 50 ml. of blood. The final concentration of citrate is 1.0 per cent.) Larger amounts of blood (300 to 500 ml.) may be obtained by puncture of the jugular vein of the sheep. The site of the puncture is first carefully shaved and disinfected and a size 16 or 18 trocar, attached by a short length of rubber tube to a suction flash, is inserted.

Citrated or defibrinated whole blood is usually added to media in the proportion of 5 per cent. Melt the agar by boiling, *cool to 45° C.*, add the blood, mix by twirling tubes (avoid bubbles) and slant or pour medium before agar solidifies. Suitable controls on the sterility of the blood must be used.

Synthetic Media. A simple synthetic medium is well illustrated by that devised by Koser:

Koser's citrate medium:

Sodium ammonium phosphate (microcosmic salt)	1.5 gm.
Potassium dihydrogen phosphate	1.0 gm.
Magnesium sulfate	0.2 gm.
Sodium citrate (crystals)	2.5–3.0 gm.
Distilled water	1000.0 ml.

Sterilize at 15 lbs. for 15 minutes.

Within the last few years great progress has been made by the application of knowledge, gained from studies of the nutritive and vitamin requirements of bacteria, to the preparation of synthetic media for pathogenic species and others whose needs for organic substances are more complex than the example given above. A good illustration of this type of complex, organic synthetic medium is one devised for the cultivation of *Corynebacterium diphtheriae* and the production of its toxin. The formula is given below in order to show the nature of such a medium.

Synthetic Medium for C. diphtheriae (Pappenheimer, Mueller and Cohen[8]).

Solution A:

glycine	.5 gm.
valine	1.0 gm.
leucine	.5 gm.
glutamic acid	5.0 gm.

methionine	.2 gm.
tyrosine	.1 gm.
NaCl	5.0 gm.
K_2HPO_4	2.0 gm.
Water	500.0 ml.

Solution B: (add to A)

cystine.......0.2 gm. (in 30 per cent HCl, minimal quantity to dissolve).

Addition 1: (Add to A after adding B)

pimelic acid	1 mg.
beta alanine	1 mg.
nicotinic acid	2 mg.

Adjust to *p*H 7.8.

Solution C: (add 0.3 ml. of this to A, after B and 1 and *p*H adjustment)

$CaCl_2$	33 gm.
H_2O	100 ml.

Boil gently 10 minutes. Filter through paper.

Addition 2: (Add to the above)

$MgSO_4.7H_2O$	.3 gm.
$CuSO_4.5H_2O$	5.0 mg.
tryptophane	100 mg.

water to make total volume of 1000 ml.

Dispense in flasks and autoclave.

Just before inoculation, to each 100 ml. add aseptically 2 ml. of

Solution D:

sodium lactate (Merck U.S.P.)	37 ml.
glucose C.P.	7.5 gm.
maltose (purified)	15.0 gm.
$CaCl_2$	0.3 gm.
H_2O to make	100 ml.

Autoclave.

The advantages of synthetic media are that they are exactly reproducible, and in some instances much less expensive and troublesome to prepare than meat infusion and peptone media. Also, they do not contain proteins and therefore have no antigenic properties when injected into man or animals. By virtue of these properties they lend themselves well to exact experimental research and to medical and commercial uses. (See sections on vitamin assay, pages 696, 699.) The great difficulty is that a solution suitable for one species is usually not suitable for another, and it is often difficult to determine the exact requirements for a given species.

Uses of Synthetic Media. A very important use of synthetic media is in the study of the requirements of various organisms for specific substances, and the converse of this, the ability of organisms to synthesize various specific compounds. For example, since *E. freundii* can grow on Koser's medium, it obviously has very marked synthetic powers, being able to form complex proteins, vitamins, fats, carb hydrates, etc., from simple minerals and sodium citrate.

By substitution of various sources of nitrogen or other elements i place of the various salts in Koser's medium, one could easily make list of forms of nitrogen, etc., available to the organism and othe forms of which it cannot make use.

Radioactive elements have come into fairly wide use in tracing th course of these elements in the synthetic processes of microorganisms For example, by furnishing *E. freundii* with a radioactive sulfur salt and by means of the Geiger counter examining various fractions* o the organisms after removal from the medium, one could determin just how the organism had utilized the sulfur in its synthetic processes

CULTIVATION OF AUTOTROPHIC BACTERIA

The complex organic mixtures referred to above serve for th growth of many heterotrophic bacteria. For autotrophic forms, th sources of *carbon,* and usually all other ingredients, are entirely inorganic. Each genus or family has certain very specific requirements especially in regard to the substance which they use as a source of energy. It is impossible to give here generally useful formulae which will support growth of all autotrophs, but one or two examples may be cited which will illustrate typical inorganic synthetic media. Thus, for the growth of certain sulfur-oxidizing soil autotrophs (*Thiobacillus thio-oxidans*), a solution such as the following will serve:

$(NH_4)_2SO_4$	0.2	gm.
$MgSO_4.7H_2O$	0.5	gm.
KH_2PO_4	3.0	gm.
$CaCl_2$	0.25	gm.
Powdered sulfur	10.0	gm.
Distilled H_2O	1000	ml.

For other species, especially certain important genera related to soil fertility (*Nitrobacter*), a solution like the following will be adequate:

$NaNO_2$	1.0 gm.
K_2HPO_4	0.5 gm.
$MgSO_4$	0.3 gm.
NaCl	0.5 gm.
Na_2CO_3 (anhydride)	1.0 gm.
$FeSO_4$	0.4 gm.
Distilled H_2O	1000 ml.

The reaction should be about *p*H 7.2.

Such media may be solidified with agar or silica gel, the choice depending on the organisms. Some are inhibited by organic substances such as agar.

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* Organic and inorganic sulfur: methionine; cystine, etc.

USE OF LIVING CELLS FOR CULTIVATION OF MICROORGANISMS

In-Vitro Live-Tissue Cultures. Practically all species of bacteria, with a few exceptions among the Spirochaetales, Chlamydobacteriales and sulfur bacteria, are known to be cultivable upon lifeless artificial media, *i.e.,* upon material devoid of *living* cells,* although some, mainly pathogens, require dead tissue or body fluids such as blood serum or ascitic fluid for optimum growth. Many will grow also upon living tissue and in so doing may cause disease, but none are *restricted* to live tissue. This is one of the properties which distinguish bacteria from the viruses and rickettsiae. The last two forms are *obligate parasites,* by which is meant that they cannot multiply outside of, or in the absence of intimate contact with living cells.

One of the simplest and most effective methods of cultivating microorganisms, especially viruses, in living cells *in vitro* was devised by Rivers in 1931 and involves what are generally referred to as "tissue cultures."[9] Many modifications of Rivers' method have been used, but in general such cultures consist of 3 main parts: (1) The suspending fluid, usually Tyrode's solution,† which furnishes mineral nutrients and glucose and to which, for some viruses such as that of yellow fever, 10 per cent serum may be added to supply protective colloids and other materials: (2) The live tissue cells, which may be chick embryo, mouse embryo, skin, liver, testis, kidney or whatever is desired. This material is obtained aseptically and perfectly fresh, minced with sterile scissors in sterile dishes and added to 10 ml. of the Tyrode's solution in 25 ml. flasks, about 0.2 gm. of the minced tissue being used for each flask. (3) The inoculum, which is added in various forms: Tyrode's fluid from another tissue culture, infected blood, bits of infected tissue, etc. The flasks are closed to prevent evaporation and are incubated in the usual manner.

Cultivation in Chick Embryos. One of the great mysteries of biology concerns the susceptibility of embryonic chicks to many diseases, bacterial, rickettsial and virus, to which the mature chick, at the moment of hatching, becomes wholly and solidly immune. What change occurs in the chick at the time of maturation and hatching which makes its tissues entirely insusceptible to organisms which, a few hours or days previous to hatching, would have multiplied unimpeded within the embryo chick or its fluids and membranes, is not known. We may

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* Erythrocytes may be regarded as lifeless since they carry on no known metabolic processes in bacteriological media.

† Tyrode's solution: (Compare with media used for autotrophic bacteria)

NaCl	8 gm.	NaH_2PO_4	0.05 gm.
KCl	0.2 gm.	$NaHCO_3$	1.0 gm.
$CaCl_2$	0.2 gm.	Glucose	1.0 gm.
$MgCl_2$	0.1 gm.	H_2O to	1000 ml.

suppose that a complete alteration in metabolic processes takes place immediately on hatching. For example, marked change in oxidation and reduction must occur with the beginning of lung function, and there is a definite change in body temperature. Exactly how these affect the resistance of the chick to the invasion of microorganisms is, however, unexplained. It may well be that complement, which is absent from the embryo, is the deciding factor. The important fact remains that the fluids, membranes, yolk sac, and tissues of partly matured chick embryos serve as excellent culture media for a great many microorganisms. A very complete discussion of all phases of the method, technics, applications, etc., will be found in the bibliography.[10, 11]

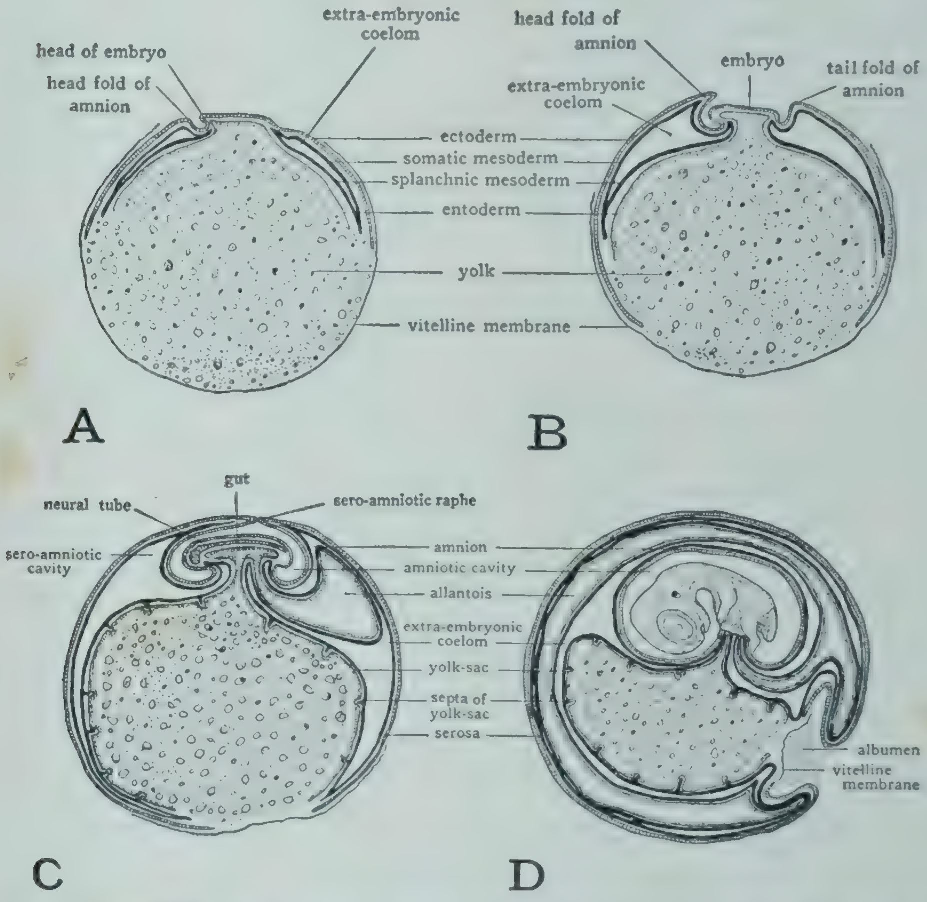
extra-embryonic coelom
head of embryo
head fold of amnion
head fold of amnion
embryo
tail fold of amnion
extra-embryonic coelom
ectoderm
somatic mesoderm
splanchnic mesoderm
entoderm
yolk
vitelline membrane
A
B
gut
neural tube
sero-amniotic raphe
sero-amniotic cavity
amnion
amniotic cavity
allantois
extra-embryonic coelom
yolk-sac
septa of yolk-sac
serosa
albumen
vitelline membrane
C
D

Fig. 74. Schematic diagrams to show the extra-embryonic membranes of the chick. (D, after Lillie.) The embryo is cut longitudinally. The albumen, shell membranes and shell are not shown; *A* embryo early in second day of incubation; *B* embryo early in third day of incubation; *C* embryo of five days; *D* embryo of nine days. (Patten, "The Early Embryology of the Chick," The Blakiston Co., publishers.)

Chick Embryology. For the student unfamiliar with the embryology of the chick the following remarks may be helpful. The egg is originally a minute cell in the ovary of the hen and contains a tiny speck of yolk which grows larger in the ovary until it is about 1.25 cm. in diameter. The yolk is nutrient substance. It is enclosed in a membrane called the vitelline membrane. The cell, with its yolk, is released from the ovary and passes through a tubular structure, the oviduct. Here it receives several coatings of albumen (the "white" of the egg) in which it floats. It is fertilized in the upper part of the oviduct. Later it receives the tough shell membrane and, in the uterus, the calcareous shell is added and the egg is then laid. Thus fertilization and the earliest stages of development of the embryo occur before the egg is laid. Under normal conditions of incubation hatching will occur in about 21 days. The early embryonic tissues consist of layers of cells which grow at the expense of the yolk and extend around and later engulf the yolk, lying upon its surface and partly covering it. From these tissue layers in great part are formed the body cavity walls and organs of the mature chick. The peripheral portions of the embryonic tissue layers become folded off and grow to form the embryonic membranes.

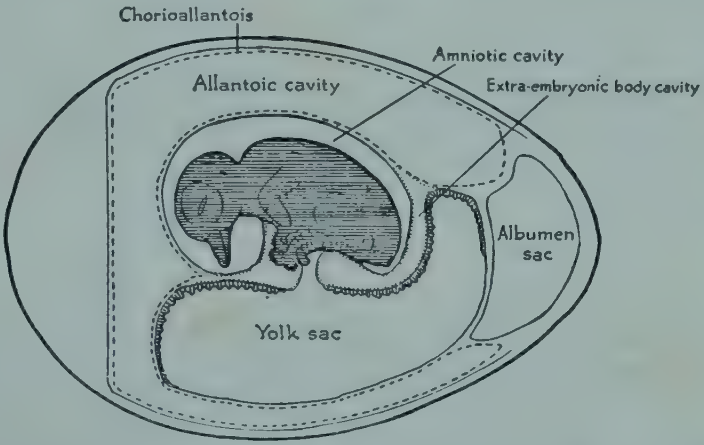

Fig. 75. Semidiagrammatic sketch showing full development of membranes and cavities in 12- to 15-day chick embryo. For clarity, the extra-embryonic cavity is shown as a real space instead of a potential one. (Beveridge, W.I.B., and Burnet, F. M., The Cultivation of Viruses and Rickettsiae in the Chick Embryo, Special Report Series, No. 256, Medical Research Council, London, 1946.)

The *chorionic* and *amniotic* membranes form about the embryo from extensions of the above mentioned sheets of cells. These membranes become very extensive and intricately folded and reflected around and over the embryo and several different embryonic cell layers grow together. Afterward the chorion and the walls of a sac developing late in the life of the embryo and called the *allantois* fuse to form the chorioallantois, or chorioallantoic membrane, which envelops the whole content of the egg. It is this that lies directly below the shell membrane and which is seen as a highly vascular area when the shell is opened. It has an important role in the respiration of the embryo inside the shell. It is left behind when the chick hatches.

Only a course in embryology could make clear to the student the whole mechanism by which the embryonic membranes are formed but the accompanying diagrams (Figs. 74 and 75) may convey some idea of their relation to the embryo proper.

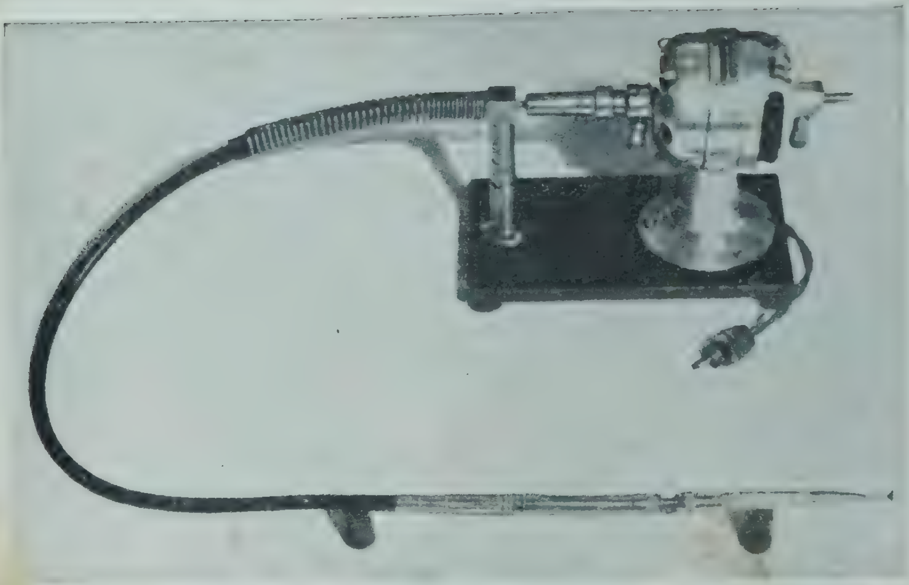

Fig. 76. Motor, flexible shaft and carborundum disc. (Goodpasture and Buddingh, Am. J. of Hyg., vol. 21.)

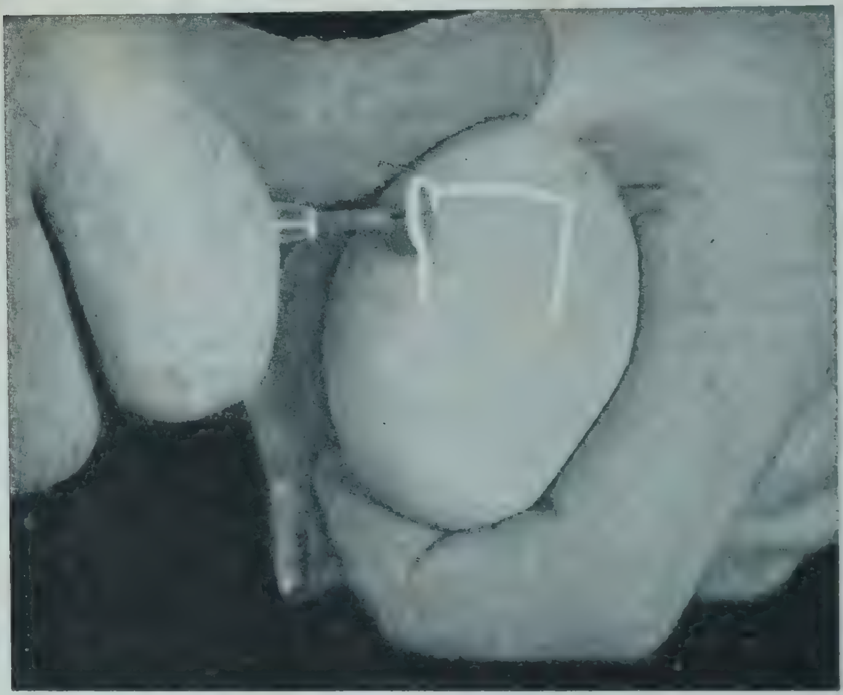

Fig. 77. Cutting window in shell with rotating carborundum disc. (Goodpasture and Buddingh, Am. J. of Hyg., vol. 21.)

Inoculation. Fertile eggs must be used. They are incubated as for hatching until the embryo is well developed (from five to fourteen days). To expose the chorioallantois for inoculation, the egg is laid on its side. It may be held in place by spring clips or by pressing it gently into modeling clay ("Plasticine"). The upper surface is then cleaned and disinfected with a weak tincture of iodine and an area about 1 cm. square outlined on the shell. The *shell* is then cut by means of a dentist's fine carborundum disc on a flexible shaft, rotated by a dental motor (Figs. 76 and 77). The square of cut shell is then gently lifted off with a sterile forceps, exposing the shell membrane immediately underneath. Before piercing the shell membrane a tiny hole is made in the shell at the large end over the air sac and slight suction is applied by means of a small rubber bulb. The shell *membrane* is then pierced, the chorioallantois drops away from it, and the contents of the egg fill the air sac leaving space under the window cut in the shell. The shell membrane may now be torn off against the cut edge of the shell like paper, leaving the vascular embryonic membranes exposed for inoculation.

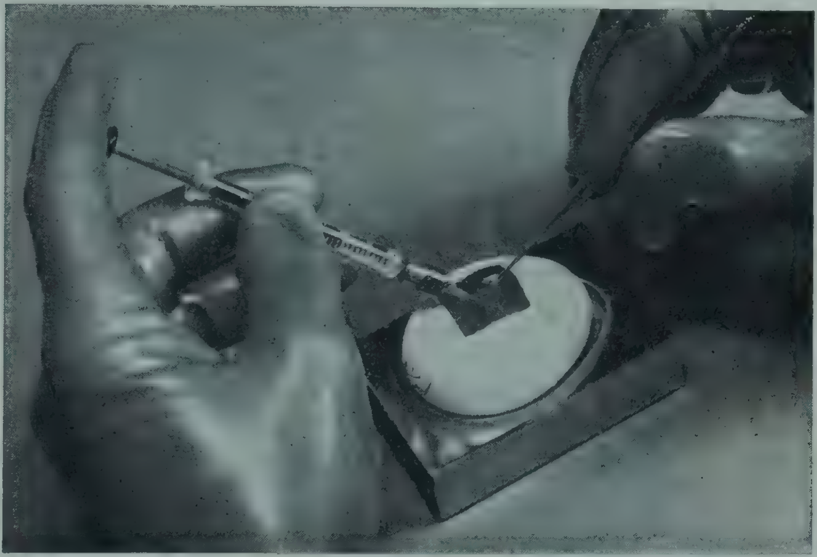

Fig. 78. Egg inoculation. Injection of filtered throat washings, from an influenza patient, into the amniotic cavity of a nine day old chick embryo. After twenty-four to forty-eight hours incubation under aseptic conditions, the amniotic fluid and lungs of the embryo are tested for presence of virus by animal passage and other means. (Therapeutic Notes, April, 1942, Parke, Davis & Company.)

This is done simply by applying the inoculum with a loop, a dropper, or other gentle means. The hole in the shell is surrounded by sterile melted petrolatum or paraffine and immediately covered with a sterile coverslip of glass, cellophane or gummed transparent plastic strip. All

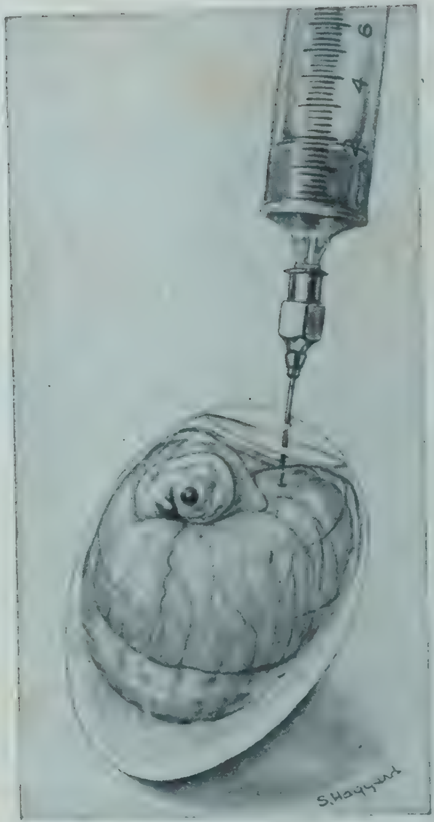

Fig. 79. Inoculation of yolk sac through small hole in end of egg. (Therapeutic Notes, October, 1942, Parke, Davis & Company.)

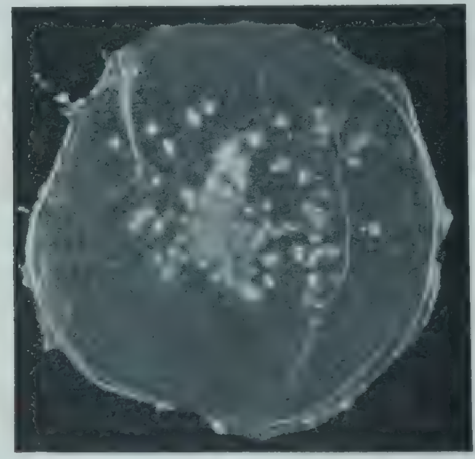

Fig. 80. Infectious myxomatosis of rabbits (3 days). (Beveridge, W. I. B., and Burnet, F. M., The Cultivation of Viruses and Rickettsiae in the Chick Embryo, Special Report Series, No. 256, Medical Research Council, London, 1946.)

holes must be stopped by whatever sealing agent is used. The egg may then be incubated as for hatching.

Modifications of this method of inoculation include injection through the membrane directly into the amniotic and chorionic cavities, yolk sac, embryonic tissue, etc. (Fig. 78). For some of these purposes a single tiny hole in the shell is all that is necessary (Fig. 79).

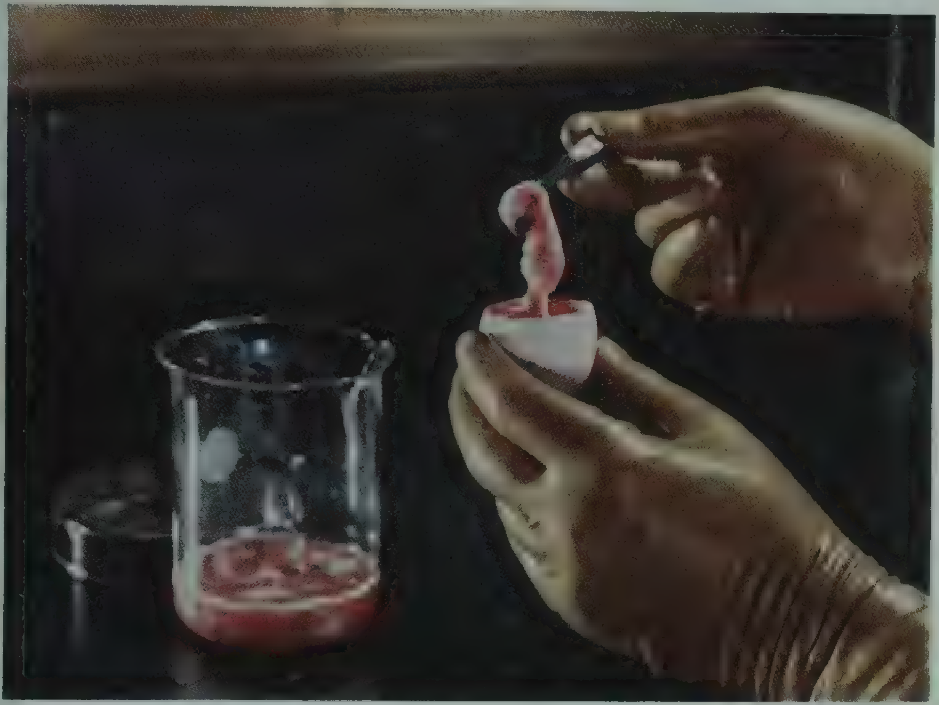

Fig. 81. The infected chick embryo and membranes (above) containing virus are removed from the egg under aseptic conditions. (Therapeutic Notes, April, 1942, Parke, Davis & Company.)

Observations of lesions (Fig. 80) developing on the chorioallantois may be made through the coverslip and material may be removed for subculture if due care is taken to avoid contamination (Fig. 81).

Chick embryos may be used for the study of many different organisms. There are reports on the cultivation of gonococci, meningococci, *Brucella, Pasteurella, Borrelia,* streptococci, staphylococci, *C. diphtheriae, E. typhosa,* fungi, etc. They are particularly used in the propagation of viruses and rickettsiae, which will not grow in lifeless media. Eggs offer the advantage of economy, availability, constant accessibility for observation of the growth and its effects, excellent opportunity for the study of tissue affinities of various organisms, antitoxins, poisons, and many other problems and phenomena.

Many saprophytic microorganisms are able to multiply as well in the fluids of the chick embryo as in the culture tube. The embryo is usually killed and destroyed by such contaminants. Extraneous contamination with such organisms from the air, shell dust, implements, or from contaminated inocula such as feces or saliva, is therefore an ever present source of error in the use of chick embryos. It is now customary to mix small amounts of penicillin and streptomycin or sulfonamide drugs with such materials before injection. These agents control bacterial contaminants but permit the growth of viruses and rickettsiae since these are not affected by the drugs.[12, 13, 14] (See Chapters 46 and 49.)

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Chapter 8

GROWTH AND VARIATION OF MICROORGANISMS

REPRODUCTION

Binary Fission. Reproduction by the organisms of the class Schizomycetes seems to be solely by means of transverse division of each cell into two equal cells. This is called simple or binary fission (Fig. 82). In bacteria, since we have no definite knowledge of the nucleus, chromosomes or genes or of how nuclear material is arranged inside the cell, nothing is known of mitosis in these organisms. Hereditary characters appear to be mediated by some means other than chromosomes, although structures and phenomena implying the presence of genes have been described. Even nuclei and chromosomes have been described but most of these descriptions are capable of other interpretations.

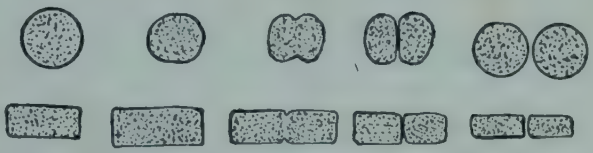

Fig. 82. Diagrammatic representation of binary fission in a coccus and in a bacillus.

Bacterial Multiplication by Means Other than Binary Fission. Other forms of multiplication of bacteria, especially by means of intracellular bodies, variously called gonidia, microcysts and regenerative granules, have been described. In these types of reproduction, multiplication may be regarded as resulting from divisions of the cell material which occur without the participation of the cell wall, so that all of the new organisms are contained within the original cell wall and are necessarily very tiny.

There is no convincing evidence that such minute bodies occur in relation to the reproduction of bacteria. In attempting to demonstrate them, one is very apt to be misled by the presence of volutin (food) granules, fat droplets and, in dead or dying cells, of globules of coagulated protein. Diffraction and refractive effects and minute granules are much enhanced in bacteria due to the acute curvature of their minute surfaces. These optical effects are undoubtedly a source of error in such studies. Even the electron microscope can do no more than cast shadows, the interpretation of which is not certain. Perhaps pha microscopy can elucidate this matter.

Sexual Multiplication of Bacteria. The question of the existence o sexes among bacteria is a much debated one. Morphological evidenc of the existence of sexes, as inferred from the apparent fusion of cell the formation of protrusions and of bodies resembling zygospores an so on, is very unreliable because of various optical limitations o microscopes, even electron microscopes. Such protrusions and zygo spore-like bodies undoubtedly appear, but there is no necessity fo regarding them as sexual or reproductive bodies. Although many persuasive arguments have been advanced one could wish for more conclusive proof. From an evolutionary point of view, sex in bacteri would be an anachronism. According to Coulter, ". . . sex is not an essential feature of reproduction [in the plant kingdom]. Historically it was the last method of reproduction attained among plants, and when it appeared it did not replace older methods [fission] but was added to them. . . . in the evolutionary sequence of plant groups the sexual cells appear for the first time far above the most primitive known plants . . . [bacteria]. . . the beginnings of sexual cells are seen among both the green algae [as *Ulothrix*] and the brown algae. . . ."[1] Further on in this chapter we shall discuss evidence for sexes in bacteria based on apparent crossing over of heritable characters from one variant to another. Here again, the facts are capable of other interpretations (see page 177).

BACTERIAL POPULATIONS

The numbers of bacteria or "populations" present in various materials depend on a variety of factors, among which are the quantity of available moisture, nitrogen, carbon, oxygen and other essentials of nutrition; the temperature, *p*H and other physical factors; the species of organisms; the presence of other organisms antagonistic or unfavorable to, or competitive with, the organism in question, the age of the population, and so on. Under the vicissitudes of natural conditions, any or all of the above factors may change irregularly and consequently the numbers of a given organism may vary irregularly. In an "ideal" situation, however, such as a pure laboratory culture under uniform, optimal physical and chemical conditions, the numbers of organisms present vary in a perfectly regular and predictable way in accord with general biological laws.

As an illustration we may select a culture flask containing 50 ml. of sterile infusion broth.

The Enumeration of Bacteria. Let us introduce, by means of a sterile pipette, a drop of fluid containing 10 cells of a common bacterium of the intestinal tract known as *Escherichia coli*. Let us assume that these are from an inactive or "dormant" stock culture held for weeks in the refrigerator. The new culture is held at 35° C. The problem before us is to measure the population at regular intervals by counting the numbers of living cells present. The numbers present at the different periods are then plotted in relation to time, and a "growth curve" is obtained. This may be accomplished by several methods.

None of the methods is exact. One, the hemacytometer method, is to place a minute drop of the fluid in a tiny, shallow, rectangular, glass vessel (hemacytometer) partitioned off by ridges into regular cubical chambers. By counting the individual cells in each chamber under a microscope, and knowing the volume of the chamber, the numbers of organisms per ml. may be computed. This is a total amount of live *and* dead organisms.

Another procedure is to smear an exact volume of the culture over an exact area on a slide, stain with methylene blue and count a known portion of the total area. Knowing from previous measurements (by means of a stage micrometer) the diameter of the microscopic field, it is easy to calculate the numbers of organisms per ml. of culture. However, no distinction is readily made between living and dead organisms.

Still another method is to place a fixed volume, say 10 ml., of the culture in a tube having a narrow, hollow, cylindrical column projecting from the bottom and graduated in mm. The organisms are packed into the column by centrifugation at a standard speed and time and their total volume read on the graduated scale. From a knowledge of the average volume of the individual cells an *estimation* of numbers is possible. This also is a total "count."

If 1 ml. of blood and 1 ml. of culture be well mixed and a stained smear of the mixture prepared, an *estimate* of the numbers of bacteria may be obtained by counting both blood and bacterial cells in a certain number of fields and noting their relative proportions. Knowing that male human blood contains about 5 million erythrocytes per cubic millimeter an estimate of the numbers of bacteria is merely a matter of arithmetic. This is a total count.

A widely used technic is turbidometric, *i.e.,* measurement of the turbidity in the fluid due to the accumulation of cells in it. This actually gives no real count of the organisms at all. Turbidity readings may be standardized in terms of numbers of cells by hemocytometer counts. A measured volume of the culture is placed in a special, clear, glass tube of known diameter. This is interposed between a source of light and a photoelectric unit which is attached to a galvanometer. The reading on the galvanometer depends on the interference with the passage of light from the unit source, due to the cells suspended in the tube. The method is subject to errors due to variation in size and shape and clumping of the cells, as well as different degrees of translucency of various species and other materials in cultures. However, the method is one of the quickest, simplest and most accurate. Photoelectric turbidimeters are available on the market.

The method of serial dilutions is widely used in water and milk bacteriology and other fields. Into tubes of broth are placed 1-ml. quantities of the sample, diluted in decimal, 4-fold, 2-fold or other convenient series. After incubation, numbers of organisms (capable of growing under the conditions provided) are recorded by noting presence or absence of growth. For example, in a 10-fold dilution series suppose there is growth in the tube which received the 1:1000 dilution but no growth in the tube receiving the 1:10,000 dilution. Then there are between 1,000 and 10,000 organisms per ml. of culture. This is spoken of as the *indicated number*. But this is not a very exact estimate. It measures *only live cells* viable (capable of growth) under the conditions provided.

Most Probable Number. In the above example there *must* be 1,000 organism but there *may* be, theoretically, any number up to 9,999 per ml. in the sampl What is the true number? This cannot be stated. However, mathematicians hav shown that the number *most probably* present may be calculated if the result from duplicate or triplicate simultaneous determinations are known. They hav prepared tables showing the *most probable number* indicated by all possibl combinations of results in such series. These are much used in sanitary examination of water. Tables are found in "Standard Methods for the Examination o Water and Sewage,"* with directions for use. It is to be borne in mind that thes are *most probable* numbers; not necessarily *exact* numbers.

The Colony Count. In this discussion we will describe the plate-count or colony-count method because it is a widely applicable one

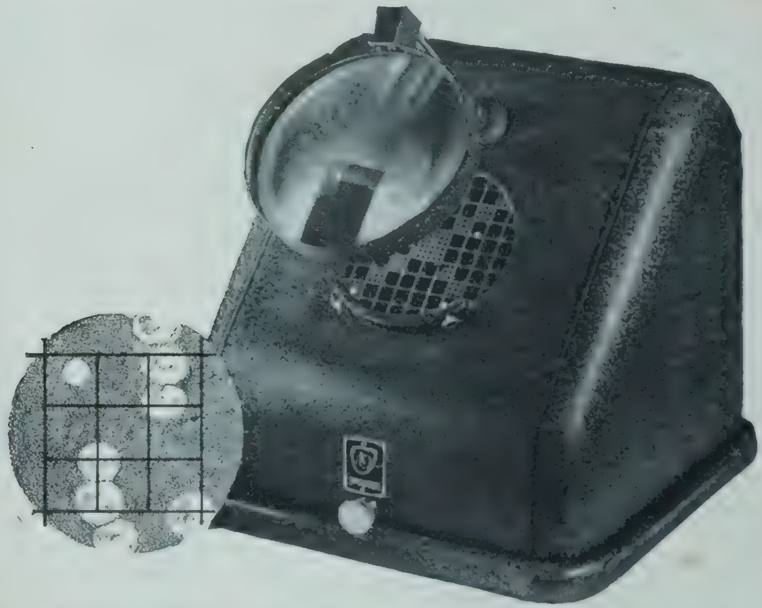

Fig. 83. Quebec colony counter. New darkfield model. Provides practically uniform and glareless oblique lighting, with darkfield effect, over entire surface of culture dishes up to 100 mm. diameter. Culture dishes are supported on a projecting ledge fitted with centering screws. Colonies in highly colored media can be counted conveniently by placing a white or colored paper in the recess of the light stop. (Courtesy Spencer Lens Company, Buffalo, N. Y.)

used daily for determining approximate numbers of bacteria in milk, water, soil, foods, etc.

At any desired moment, exactly 1 ml. of the culture is withdrawn from the flask with a sterile measuring pipette and is transferred to a sterile Petri dish. Immediately afterward, about 15 ml. of nutrient (meat extract) agar, previously melted (and cooled to about 40° C. so as not to kill the bacteria), are poured into the dish and the culture thoroughly mixed therewith by a gentle horizontal rotation of the dish. In a few minutes the agar will have resolidified. This plate-culture is held in an incubator at about 35° C. for twenty-four hours and is then examined for the presence of *colonies,** distributed throughout the agar (Fig. 83). Each of these represents, *theoretically,* the progeny of a single cell which was in the original inoculum and was imprisoned in or on the agar at that point. Actually several organisms, if stuck together in a clump, will give rise to only a single colony so that the colony count does not give a wholly accurate enumeration of the live, *individual* cells present in the material under investigation. However, the errors in the plating method are known, in a general way, and within its limitations the plate count is one of our most useful means of enumerating bacteria. It is very widely used and should be fully understood at this point.[2] It measures only organisms viable under the conditions of growth provided.

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* American Public Health Association, New York, 9th ed., 1948.

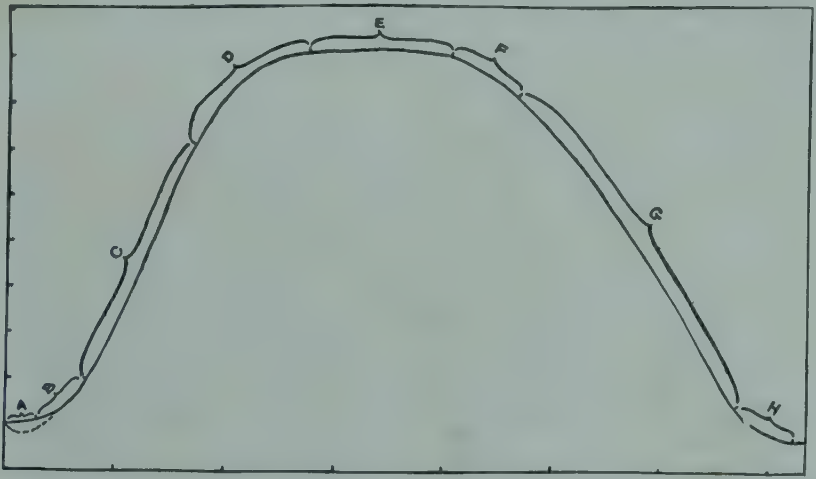

Fig. 84. Growth curve of hypothetical culture of *Escherichia coli*. For explanation see text.

Determination of Growth Curve. Let us suppose that we make plate counts on our culture of *Escherichia coli* at rather frequent intervals (two hours) at first, and plot the numbers of colonies (roughly, live organisms per ml.) against time. We may continue this until no further significant changes occur, and at the end of this time a curve will have been obtained which will look somewhat like that seen in Figure 84.

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* It is often desirable to examine the plate by means of a special illumination and a 2 × or 3 × magnifying glass so as to make even the smallest colonies visible. A convenient device for this purpose is seen in Figure 83. The criss-cross lines are for the purpose of guiding the eye in making the colony count.

As the number increases toward several millions per ml., the 1 ml. of culture removed for the plate count is diluted so that plates are obtained which show only about 50 to 300 colonies. This is easy after a little experience. It avoids lethal crowding of the colonies and separates the colonies so that counting is easier. The number of colonies, multiplied by the dilution, gives the "number per ml."

A totally different type of curve would be obtained if we were to count the bacteria in the fluid by means of one of the total count methods described above. A curve showing *total* counts as compared with viable counts is seen in Figure 85.

Phases of the Growth Curve. The curve shown in Figure 84 has several portions which deserve discussion. These are shown by brackets and labels in the figure. Portion *A*, usually called the *latent* or *initial stationary phase,* represents a period during which the dormant organisms are probably imbibing water, becoming adjusted to the new environment, and so on, much as might occur when a dormant tree is set out in the spring. There is no immediate growth (increase in numbers). The dotted line indicates that some few of the cells may actually die off during this period, only the more vigorous going on to multiplication which soon begins to show itself by the rising inflection in portion *B* which is properly called the *phase of accelerated growth* but is often referred to as the *lag phase*. The first two phases together also are often called the lag phase.

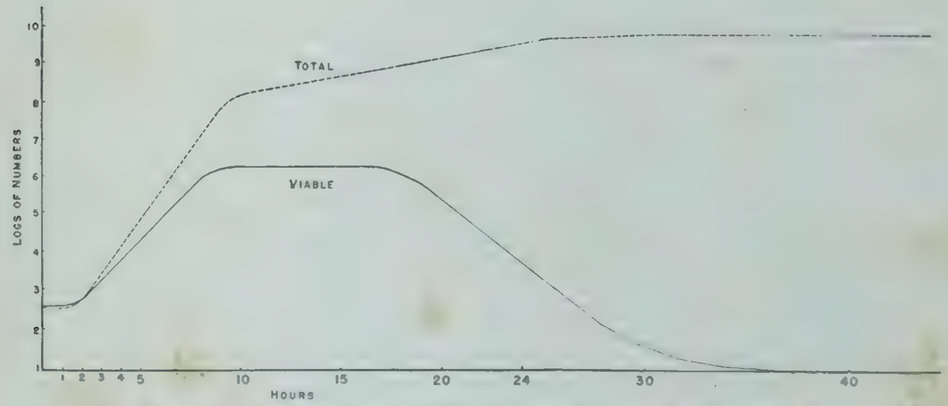

Fig. 85. The relation of total to viable counts of bacteria.

When very small inocula of certain species are transferred to media like pure serum or egg, consisting of native proteins only, with none of the simpler products of protein hydrolysis present which can diffuse into the cell, the cells cannot initiate growth, for, presumably, they have nothing but unavailable, undigested proteins on which to grow. In the absence of growth, they cannot form the necessary proteolytic enzymes. If larger inocula are used, probably some small amounts of protein hydrolysis products, or of the enzymes themselves, are carried over from the previous culture and allow a few cells to start growth, thus producing enzymes which hydrolyze the native proteins and permit further growth. Also, in the larger inocula, there may be enough dead cells so that, as they disintegrate, sufficient enzyme is liberated to initiate growth. This may, in part, explain the initial stationary phase. The mutual stimulation of a number of cells to growth has been called "allelocatalysis." However, in non-protein media, also, the same phenomena of dormancy and lag are observed. No adequate explanation is available.

During the phase of accelerated growth the time required for each cell to divide is decreasing, fission occurring more and more rapidly as the organisms become adapted to the new culture medium. Eventually they reach their maximum rate of fission, which may become so rapid that the number of organisms doubles every twenty to thirty minutes. Fission rate varies very greatly with different species and under different conditions of growth. Tubercle bacilli, for example, probably divide only about once a day at the highest rate of growth.

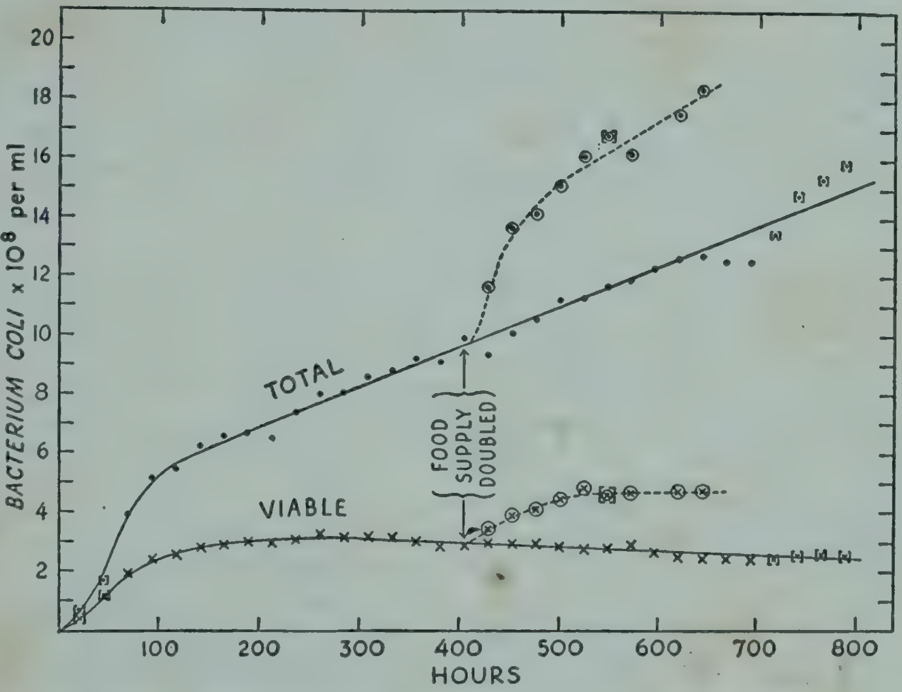

Fig. 86. Mean growth curves for bacterium coli on constant food supply of 15.2 Mg. Difco broth per hr. at 35 C. (Jordan, R. C., and Jacobs, S. E. in J. Bact., vol. 48.)

The logarithms of the numbers of live organisms counted at short intervals, plotted against time, produce a straight line as shown in Figure 84. This period is spoken of as the *phase of logarithmic increase*. Were this to continue uninterrupted, the culture would become a solid mass of bacteria in a few hours. A single cell of *Escherichia coli* allowed to continue such growth for a year or so, would produce a mass weighing more than the sun!

During this phase most of the cells are young and biologically active. If a subculture is made from the flask to a new flask of warm, sterile broth, growth continues at the logarithmic rate; there is no lag or dormant phase. The lag and dormant phases are reproduced in more or less degree in subcultures from any other phases of the growth cycle than the logarithmic. The young logarithmic cells are also apt to be more easily killed or adversely affected by various inimical agents. Biochemical peculiarities used for identification of organisms are usually most manifest during this period.[2a] (See Fig. 86.)

Within a few hours after the commencement of the logarithmic phase, the organisms begin to encounter difficulties. Food begins to run out, poisonous waste products accumulate, respiration becomes difficult, and the cells become so crowded together that they jostle one another and this interferes with their proliferation. The rate of fission begins to decline and the older organisms die in increasing numbers, so that the increase in number of live cells slows, as shown in the portion of the curve labeled *D*. This is spoken of as the *phase of negative growth acceleration.*

Immortality (?) of Bacteria. It is interesting to speculate upon the relationships between the age of cells, their multiplication and their continued existence without fission. Let us compare the fate of two cells just produced by the fission of their "parent." Under favorable conditions one undergoes certain unknown physical and chemical changes which are the equivalent of "maturation" and, after a time, undergoes fission like its predecessor. What is the age of the new daughter cells? Are they, physiologically, infantile cells, or have they the age of their parent cell? What mechanism determines when they shall again undergo the mysterious changes of maturation and in turn become young again by the process of fission, thus theoretically propagating indefinitely and never growing old or undergoing senescence? Are they, by virtue of continual renewal of youth, immortal?

The other of our two original cells fails to reach a state in which fission occurs, and remains intact. What is its physiological age status? If transferred to a new culture it may begin multiplication at once and become "young" or it may die. It may die even if left in the original culture. Does it die of "old age"? The explanation of its fate is quite obscure. Evidently chronological age and physiological age may be very different matters among bacteria.

Final Phases. Eventually (the time depending on the temperature, the size of the flask and volume of fluid, the composition of the medium and numerous other factors), the number of cells dying balances the rate of increase, and the total population remains unchanged for a time. This phase, the *maximum stationary phase*, is shown at *E*.

As conditions become more and more inimical to the bacteria, they grow more slowly, and death overtakes them in ever-increasing numbers, as shown at *F*, the phase of accelerated decrease or *accelerated death phase.*

This progresses into the *logarithmic death phase* (*G*), during which decrease in number occurs at a regular, unchanging rate.

Finally, conditions begin to reach an equilibrium such that both rate of death and rate of increase tend to balance each other again at a very low population level, and the *phase of readjustment* (*H*) and the *final dormant phase* (*I*) are attained. Complete sterility of the culture may not ensue for weeks or months, depending on the kind of organism.[3, 4, 5, 6]

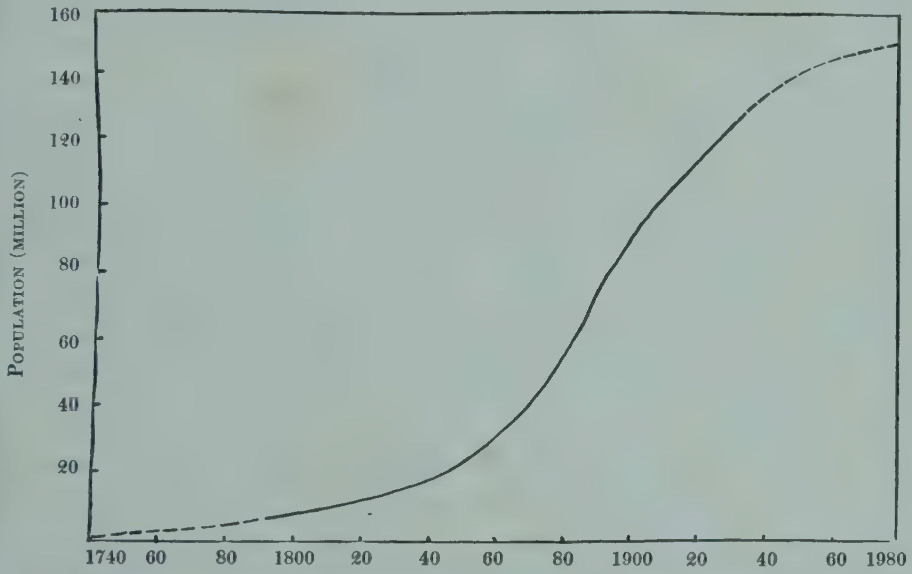

Fig. 87. Approximate population curve for the United States. Compare with the first portion of the curve for a bacterial population. What will happen to the U. S. population curve by A.D. 2000? Will a war affect it?

Factors Affecting Growth Phases. The form of the growth curve may be affected by many factors. For example, if the culture be suddenly plunged into icewater, the curve at once ceases its upward trend, remains flat for a time and then begins to decline. If held at 22° C. instead of 35° C. (for *E. coli*) the rise in the positive phases is much less abrupt and much more extended. Other factors such as *p*H, concentration of food, and so on, have their effects also. If the toxic growth products in the culture be slightly diluted and new food in solution be added during the maximum stationary phase, growth immediately resumes at a logarithmic rate for a while. If a continuous supply of food solution (and dilution of toxic products) be continued, the maximum stationary phase is longer. If food is then discontinued, the phase of logarithmic death sets in very soon[7] (Fig. 86).

The growth curve is of interest in that it is representative of all normal population curves, plant, animal and human. By a normal curve is meant one which is not disturbed by special factors such as excessive immigrations or emigrations, wars or natural catastrophes. The population curve of a new settlement, for example the United States (Fig. 87), has a similar form, but there are factors, such as wars, plagues, immigrations, emigrations, etc., which may cause irregularities to appear from time to time.

It must be pointed out that the curves shown in Figures 84 and 87 are not strictly comparable, since in the former *logarithms* are plotted against time while in the latter *actual numbers* are used. Were the same system used in Figure 84 as in Figure 87, Figure 84 would have to be some *1200 miles* high to accommodate the huge numbers on which the curve is based and it would go up and down precipitously as compared with the curve in Figure 87, since one is scaled in hours, the other in periods of 20 years. Nevertheless the same general principles underlie the form of both curves.

VARIATION OF MICROORGANISMS

1. Period of Unlimited Pleomorphism. For many years following the discovery of microorganisms by Leeuwenhoek there were few clear ideas as to the true structure and taxonomic relationships of the "animalcules." Some workers regarded all microscopic forms of life as various manifestations of one and the same species. It came to be generally believed that they could, and did, take a great variety of shapes and sizes and could change their color, and growth form and other properties. This was called *pleomorphism* (*pleo* = many; *morph* = form).

Not until Koch and his co-workers developed pure culture methods (1882) based on plating and colony fishing did this fog of confusion begin to clear away.

However, in spite of Koch's classical work there occurred within the last three decades a rather lively contest of experimental data reminiscent of the controversy over spontaneous generation. It was stated by one group of workers that the action of an ultramicroscopic organism parasitic for bacteria (bacteriophage, see page 844) and certain other influences, such as rapid plate-to-plate transfer (Hauduroy technique), would induce the appearance of virus-like forms which then transformed themselves into very different bacterial species. Only after a series of experiments showing that these "new" and "different" species were eliminated by the simple expedient of improving the aseptic technique of the experiments, to eliminate contamination, did this notion subside.[8, 9, 10]

These extreme ideas were the last of the doctrine of pleomorphism that had its roots in impure culture techniques.

2. Period of Strict Monomorphism. As a result of extremely careful and painstaking experimental work and observation Ferdinand Cohn, a German scientist contemporary with Koch, stated, about the year 1872, that he believed bacteria to be much less variable than had previously been thought. This was a great advance from extreme pleomorphism and accordingly aroused a storm of protest. When Koch studied bacteria a few years later he had the advantage of exact, pure culture methods and he kept transferring his cultures on the same sort of media under very uniform conditions so that they tended to remain uniform in character. He, better than anyone else at that time, could appreciate the deceptive role of contaminants in producing apparent changes in bacteria and, as a result, he arrived at the conclusion that the doctrine of pleomorphism was largely the result of contamination. There then arose, among his pupils, the doctrine of strict monomorphism. It was then taught that bacteria *do not vary at all;* at most in insignificant details.

3. Period of Rational Pleomorphism. Later, irrefutable data began to appear showing that bacteria do indeed vary in many characters. As a result of technically impeccable experimental work by scores of microbiologists in which no question of contamination arises, a rational position has finally been reached.[11, 12]

The truth lies somewhere between strict monomorphism and extreme pleomorphism, and might be termed "limited pleomorphism," or *oligomorphism* which means, literally, "few forms." Probably no modern bacteriologist denies that bacteria vary and that they do so within fairly wide limits. There can be no question that variations in size and shape of cells, motility, capsules, pigment, and other characters occur, often as a result of what seem to be very small irregularities in time and temperature of incubation, age of culture, composition of medium and other environmental factors. Some of the changes appear to occur spontaneously, *i.e.,* for no *assignable* reason. Some are temporary, *i.e.,* are *variations;* others are permanent, *i.e.,* are *mutations*. Some of the basic differences between these terms will be discussed later.

Examples of Microbial Variation. For many years the best known variations were in those features of microorganisms most readily observed—size and shape of colony and cell, color of colony, motility, capsule, spore, and so on. These were also the early bases of taxonomic work. As knowledge of the less conspicuous properties of microorganisms increased, especially data on biochemical, physiological and immunological properties, other types of variation involving these properties were observed. The taxonomy of microorganisms has become correspondingly complex. It appears that almost any microbial character is subject to some variation. Only a few illustrative and common kinds of variant will be described here.

COLONY VARIATIONS

Among the most striking and important phenomena of bacterial variation are those related to the form of the colonies on solid media. There is a voluminous literature on the subject, and dozens of different variant colony forms have been described. However, there are five or six forms of colony variant which are most frequently seen and to which we shall confine our attention.

Rough and Smooth Colonies. Let us consider the colonies produced on infusion agar by a strain of a saprophytic, aerobic, spore-forming bacillus common in dust. In one series of observations the colonies ordinarily produced by the organism on extract agar in a particular laboratory were about 2 mm. in diameter, gray and translucent, but appeared in two different forms. One form of colony was perfectly smooth, moist and homogeneous, convex, circular and glistening and had regular margins (Fig. 88). It was butyrous (butter-like) in consistency. This is a well-known type of colony variant occurring in most species of bacteria and is spoken of as a *smooth* or *"S" type* of colony.

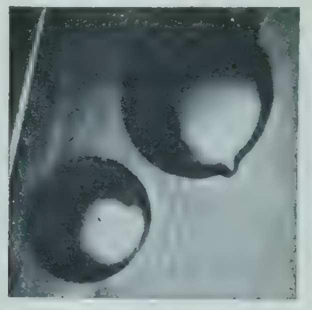

Fig. 88. Colony variant of aerobic, spore-forming bacillus. Smooth or "S" type of colony (× about 8).

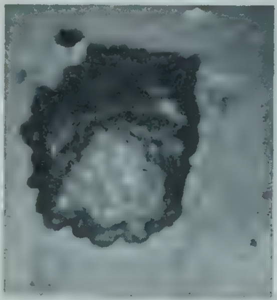

Fig. 89. Colony variant of same organism depicted in Figure 88. Rough or "R" type of colony (× about 8).

The other type of colony was not glistening but rather dull, and had rough or wrinkled surfaces, and irregular edges. It was dry and crumbly in consistency. This also is a well-known form of colony variant occurring in many species of bacteria and is called a *rough* or *"R" type* of colony (Fig. 89). It is commonly observed that the cells in rough colonies form themselves into long tangled filaments on the surface of solid media whereas in smooth colonies the cells tend to occur singly. In broth the rough growth is usually granular or flaky, often growing in a thick scum or pellicle on the surface; the smooth growth producing an even turbidity. Practically all species of bacteria vary in this way. There are often intermediate degrees of "roughness" or "smoothness."

The phenomenon of variation, especially as it relates to colony form, is often loosely spoken of as *dissociation*. However, the term "dissociation" is borrowed from the ionic theory in chemistry and should not be applied to bacterial phenomena.

Frequently S forms seem to represent a reaction to unfavorable environmental factors, though this is not necessarily always so. Contact with disinfectants, certain salts, etc., may stimulate the appearance of S forms. Among bacteria capable of infecting animal tissues, the appearance of the S form is favored by contact with the infected host which resists the infection. Virulence is thus commonly associated with the S form, and this, in turn, with the formation of a visible capsule or with analogous modifications in the surface of the organisms.

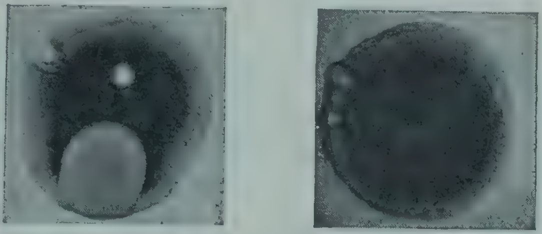

Fig. 90. Colonies of a species of spore-forming aerobic bacillus showing sectors (light areas) of nonsporulating rods of the same species. (Photographs courtesy of Dr. J. Howard Brown.) (× about 15.)

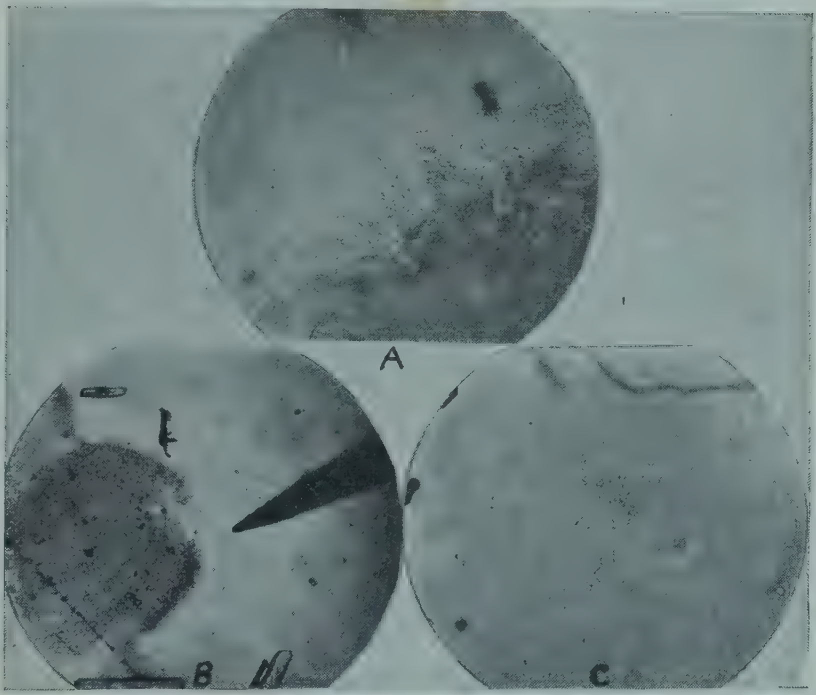

Fig. 91. *A,* Margin of colony magnified about 500 times to show sporulating and nonsporulating growth. The dark, oval granules are spores, and the dark areas in the lower right part of *A* are masses of sporulating cells or filaments of cells. *B,* The tip of the micro-manipulator needle is seen teasing apart the margin of a colony so as to separate the sporulating from the nonsporulating growth. *C,* A very young colony developing from a single, asporogenous cell. This growth is only a few (4–5) hours old and is completely invisible except with the high-dry lens. This is growth of the R or rough type.

These surface alterations, in turn, affect the colloidal properties of the cells and hence their immunologic and antigenic properties (since these are all largely surface phenomena). These alterations in the antigenic properties of organisms are of the utmost importance; for example, in the preparation of vaccines virulent

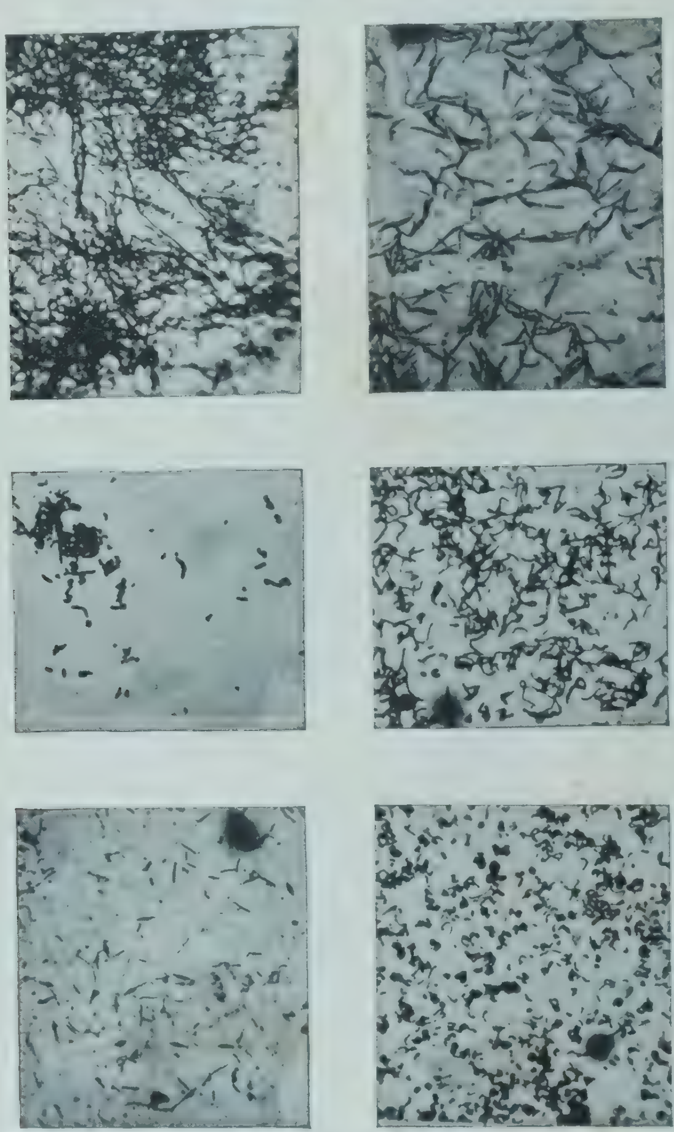

Fig. 92. Different morphological types assumed by a single kind of organism (× 900).

S forms are especially sought, since they induce protective antibody formation. In diagnostic, taxonomic, forensic or industrial studies based on antigenic structure, whole systems of results may depend on whether S or R forms are being used.

When S forms of bacteria are cultivated on artificial media in pure culture, removed from competition with other bacteria that occur in natural environments like the soil or throat, under wholly uniform, benign and favorable conditions, they frequently tend to lose their virulence and their protective surface structures and revert to the saprophytic, or possibly less highly differentiated, R form. There are exceptions to this, but in artificial cultures it is common to observe this transition.

Other differences between R and S variants are frequently noted. For example, in the spore-forming organism referred to above, colonies were frequently observed having sectors of nonsporulating cells (Fig. 90). By means of a very fine needle and an instrument called a micro-manipulator,[13] these two types of cells (sporulating and nonsporulating) could be separated in pure culture (Fig. 91). It was found, on further study, that a *single cell* of either type could produce the other, *in either the R or S form*. Thus, sex is not involved. No reason could be found to account for the variations. They *seemed* to occur spontaneously. Loss of spore formation is a common observation. In addition to this, cells of the organism being described, in different colonies often of the same type, varied so widely in size and form as to be unrecognizable as the same organism. Some of the morphological variants are shown in Figure 92.

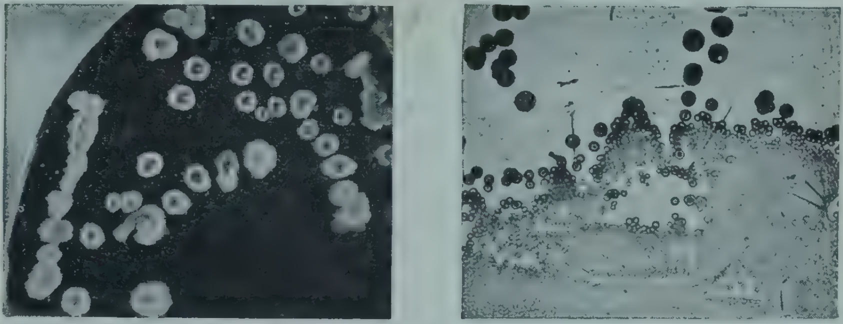

Fig. 93. Colony variants of a species of spore-forming aerobic bacillus. Compare these colony forms with those seen in Figures 88, 89. (Natural size.)

Changes in the cell form of the same general nature are of common occurrence in many species of bacteria, but are not always exactly the same as these, often being much less extreme.

Mucoid Colonies. A strain of motile, spore-forming organisms similar to those just discussed, when cultivated on infusion agar containing a little phenol, produced not only R and S types of colonies but ten or eleven other varieties wholly dissimilar from each other in appearance. Two of these are seen in Figure 93. Some of the colonies were large, viscous and slimy, like mucus. This kind of colony is common in many species of bacteria, both spore-forming and non-spore-forming, rods and cocci, and is called "mucoid" or M. The mucoid material is probably an exaggerated capsule or slime layer. It seems to be secreted by the cells in response to various external stimuli (in this case phenol) and probably results from a response of the organism to the environment (Fig. 94).

Minute or Small Colonies. Rough, smooth, and mucoid colonies are of common occurrence and are often relatively stable in character. Many other types, as has been said, may occur. The literature on the subject sounds somewhat alphabetical. There are R, S, and M types, O, H, X, and Y forms and others.

One form of a curious nature is the minute or small colony. Some workers have suggested that these colonies represent gonidial (intracellular granule) developments, hence they are sometimes referred to as G colonies. As such granules do not occur, the term G colony is a misnomer. A better term for them is dwarf or minute colonies. It is impossible to illustrate these because they are so tiny as to be almost invisible. These colony variants often tend to revert to the larger colony forms quite readily. Minute colonies may also be R, S or mucoid.

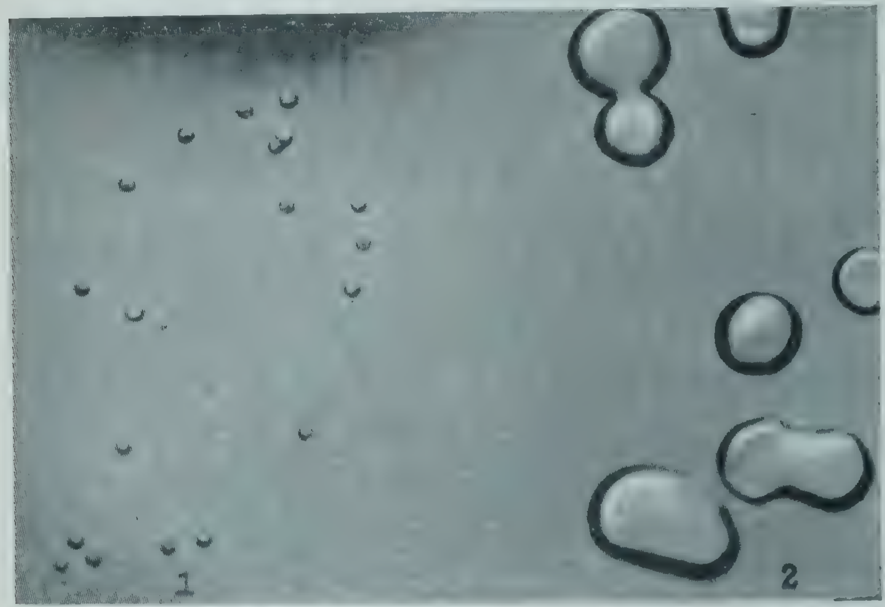

Fig. 94. (*1*) Colonies of the R variant (R36A) derived from Pneumococcus Type II. Plated on blood agar from a culture grown in serum broth in the absence of the transforming substance. × 3.5. (*2*) Colonies on blood agar of the same cells after induction of transformation during growth in the same medium with the addition of active transforming principle isolated from Type III pneumococci. The smooth, glistening, mucoid colonies shown are characteristic of Pneumococcus Type III and readily distinguishable from the small, rough colonies of the parent R strain. (Avery, O. T., MacLeod, Colin M., and McCarty, Maclyn in J. Exper. Med., vol. 79.) The photograph was made by Mr. Joseph B. Haulenbeck.

H and O Variants were first described in connection with *Proteus vulgaris*, a non-sporeforming, gram-negative, motile rod commonly found in feces, stagnant water and decaying organic matter. When cultivated on agar plates or any moist solid medium, *P. vulgaris* usually spreads over the surface quite widely in a thin, smooth, gray, translucent film, by a process of extension called "swarming." The individual cells in such growth are very motile, *i.e.*, they have flagella and swim out from the edge of the colony in the film of moisture on the surface of the agar. This form of the organism has been designated as the *Hauch* or "H" form (the German word *Hauch* meaning a film or veil).

A second form of growth is observed in which the colonies are small and discrete. In these the *Proteus* bacilli are found to be nonmotile, *i.e.*, they possess no flagella.* These are called the *ohne Hauch* or "O" form (*Ohne* = German for "without").

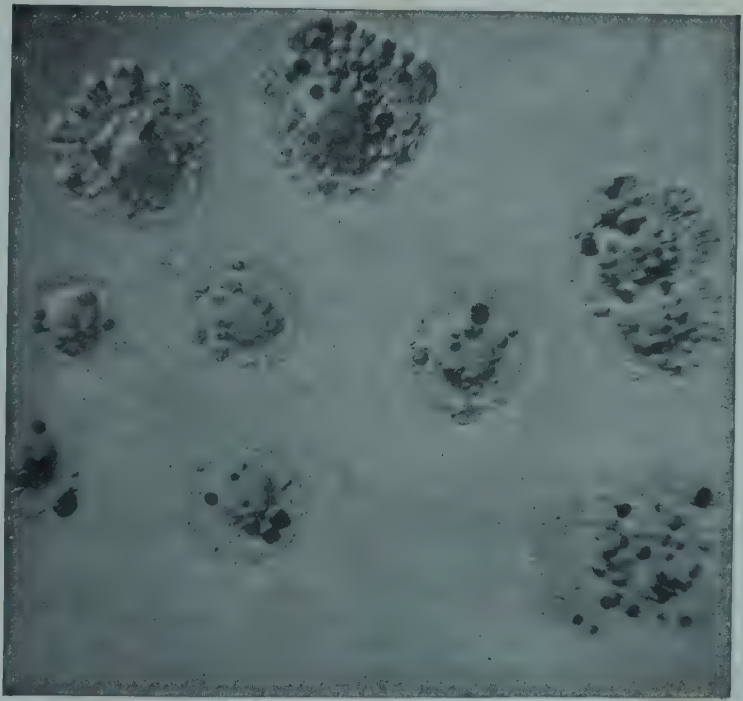

Fig. 95. Colonies of *Salmonella typhi* (Dorset strain) showing secondary or daughter colonies. Magnification × 10. (Army Medical School.)

The antigenic structures of these variants are of great interest, especially in relation to the diagnosis of typhus fever (see page 859), and also of typhoid fever and related diseases (see page 725).

Motile and nonmotile variants of other organisms are common. Although all motile variants may not have the spreading "swarming" character (*Hauch* form) of *Proteus,* they are often referred to as H forms, meaning flagellate or motile, while the *ohne Hauch* forms are called O forms because they have no flagella. As will be seen later, the term H is often used to refer to the flagella themselves or to their proteins (H proteins) and the letter O to designate the body or somatic substances (O substances) of various bacteria.

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* If, as stated by Pijper, flagella are in reality nothing but tags and shreds of capsular material, their functions in relation to motility cannot exist and the term flagella is a misnomer. But the immunological relationships will not be altered and in many respects will be much simplified.[14] Pijper's work remains to be confirmed.

Secondary Colonies. The formation of small excrescences, papillae or outgrowths from ordinary colonies of many species of bacteria, after the first growth is mature and begins to age, is a curious but common phenomenon. The outgrowths are called secondary colonies or "daughter" colonies. They may appear on the surface, develop from within, or grow out from the edges (Fig. 95). They vary in size, form, numbers and appearance. Usually the cells in secondary colonies vary from the original in many properties, both morphological and physiological.[15]

The reason for their formation is not clear. Apparently, after the original colony matures, conditions for further normal growth become unfavorable and the cells become unstable, varying in response to the changed conditions. The secondary growths may be of R, S, or mucoid types, and transfers from them may produce cultures with altered antigenic structure, fermentative properties, etc. Such variants of *Escherichia coli* are especially well known. One variety of this species, formerly referred to as *E. coli mutabile,* was so named because of its tendency to produce secondary growths with fermentative properties differing from the parent growth.

Reverse Variation. A remarkable character of bacterial variants is that they frequently revert to their original or common form. The variation that has occurred appears to be unstable or reversible. Often this reversion to original form occurs immediately on removal of the influence that induced the variation. At other times, the reversion occurs only long after removal of the stimulant. If reversion does not occur (within the period of observation) a mutation is said to have occurred. This is a new species.*

Biochemical Variants. A great many types of change have been observed in what seem to be fundamental physiological properties such as cell metabolism and synthesis of the cell substance. For example, variants occur in which there are deficiencies in ability to synthesize certain vitamins, amino acids or other substances essential to the cell. Unless the environment contains these factors already formed, the variation is what geneticists call a "lethal."[16]

Other variations include development of resistance to bacteriophage. This is often coupled with other changes, such as loss of virulence, R form, or deficiency in tryptophane synthesis.[17, 18]

Marked increases in metabolic activity or in synthetic ability, rather than deficiencies, are characteristic of still other variants. For example, it is possible to "train" organisms to utilize a carbohydrate, such as lactose, which was not originally utilized, by cultivating them constantly in the presence of lactose. Apparently a new enzyme has appeared or a latent one developed. Such enzymes are often called *adaptive enzymes.* Conversely, bacteria requiring a specific amino acid, as tryptophane, may be induced to grow in the absence of this substance. This phenomenon of adaptation to various situations by alterations in the cell is reflected in the phenomenon of drug-fastness (see page 225).

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* Or is it? Who is to say how long a period of observation of a "variant" must elapse before it is declared a "mutant?" May not some of our so-called species be merely variants which tomorrow or fifty years from now will revert to their parent type?

In most cases adaptive alteration in the organism is observed only as a result of contact with the substance involved. The question immediately arises as to whether this variation in the culture represents selection of individuals in the culture population which are especially favored by the conditions provided (as in acquired lactose-fermentation ability) or which are especially resistant (as in development of drug-fastness or resistance to high concentrations of salt), or whether the new food (or the drug or salt) influences the *hereditary* characters of the organisms, inducing the appearance of mutants or variants. Studies on the relative proportion of the resistant or more fermentative cells present in a given volume of the culture under "ordinary" conditions of growth, as compared with their numbers when in contact with the drug (or salt or sugar or other influence), gives some clue to a solution. If, when in contact with the determining influence, the altered cells occur no more frequently than when not in contact with that influence, then it may be said that the influence merely selects the resistant or favored cells from among a population in which they may normally occur but only as obscure and rare variants. If the influence causes abnormally large numbers of the new types to appear *de novo,* then it appears to induce their formation as well as to favor their growth.[19] These considerations are of more than mere theoretical interest because they bear directly on the problem of cell mutation and this on many other problems, notably that of cancer, origin of viruses, etc.[19a]

Other Variants. It is impossible in this brief space to describe all observed bacterial variants, but it may be said in passing that colonies of pigmented organisms vary in color, motile species give off nonmotile variants, virulent ones become nonvirulent, and so on.

Variations in degree may also occur. Thus, an organism producing much toxin may gradually produce less and less as successive cultures are made, yet never lose the property of toxigenicity entirely. A potent lactose fermenter may become a weak fermenter and so on. Different strains of the mold *Penicillium notatum* vary greatly in penicillin production.

Independence of Characters Which Vary. In any organism, variants of either the R or S or some other type may, when cultivated on the proper media, produce colonies of any other sort, with cells having various and unpredictable combinations of properties. Sporulation, in those species in which it occurs, is not necessarily related to either R or S type. Combinations of any characters may appear in the variants of a given organism.

It is difficult to predict what characters may be associated. Mendelian laws do not *seem* to hold in this sexless (?), chromosome-free (?) world of bacteria. This is quite different from the regular association of certain characters which takes place in higher plants and animals.

Mechanism of Variation. Among organisms in which sexes are evident and in which there are definite mechanisms of heredity, nuclei, chromosomes, genes, mitosis, etc., a good deal is known of the mechanisms of variation. Chromosomes and genes have long been known as the mediating agents in heredity of higher plants and animals. In certain molds with sexual spores, genes responsible for certain synthetic functions may be inactivated by irradiation with x-rays so that metabolically deficient mutants arise which require, ready-formed, certain vitamins and other essential metabolites which they formerly could synthesize for themselves. These new properties are hereditary and permanent in an exact mendelian manner. The number of variants induced is dependent, within limits, on the dosage of x-rays. Evidently x-rays destroy or inactivate individual genes which control certain specific synthetic activities.[16] This is clear in molds, but we have no knowledge of genes and sexes in bacteria.

If, as is common practice in bacteriology, variants of a given kind of organism having stable and permanent metabolic differences are classed as different species or even genera, then we see in x-rays a mechanism by which new species or genera may be produced.* If the environment favors them, they survive. If the environment is not favorable, the new species becomes extinct.[34]

We see in these two phenomena (production of new mutants and automatic environmental selection of the more resistant or favored) two major instruments of evolution. The development and control of evolution in the laboratory is indeed a great step in solving one of the mysteries of Nature. In such studies of cell variation appears to lie one of the most promising approaches to the control of cancer or neoplastic growths since these may represent growths of mutated cells.

Induced Variation. In bacteria, not only may the rate at which common and well recognized variants (spontaneous variants) be greatly increased by exposure to ultraviolet and x-rays, but variants not normally occurring, or at least so very rarely occurring as to be unknown, may be made to appear promptly in appreciable numbers. These are called induced variants. Differentiation between spontaneous mutations (or variations) and induced mutations (or variations) is an arbitrary matter. In regard to "spontaneous" mutations we cannot say when or why variation occurs but only that the variant type is present and continuously occurring at the time of our first observation of the culture. In the case of "induced" mutations we can say that a certain experimental influence (as x-rays) stimulates the appearance of variants at a certain time. It might have occurred "spontaneously" at a later time. There is no reason to think that "spontaneous" mutants differ fundamentally from those produced experimentally.

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* The propriety of giving species or generic status to such varieties is a difficult problem; one of the thorns in that briar patch, taxonomy. As stated by one writer, studying mutations, "Other mutants are of a type which could be classified as a new 'species' if one did not know that they originated from certain fungus cultures" (as a result of radiation).[20]

Mutagenic Agents. It is of special interest that influences, e.g., ultraviolet and x-ray, which induce mutation in microorganisms also induce variation in higher animals. Thus x-ray and ultraviolet irradiation induce the formation of biochemical variants in fruit flies as well as in bacteria. Similar effects are obtained by the use of chemicals such as the nitrogen and sulfur mustards ("mustard gas").[21] These similarities of action of various influences suggest a similarity in the responding heredity mechanism in the affected cells. If genes are the unit affected in flies and molds, then similar effects in bacteria suggest the presence of genes. This in turn suggests chromosomes, these a nucleus, and the whole heredity equipment: sexuality, with fusion of nuclei, and mendelian inheritance of characters. Morphological studies supported by the use of the electron microscope, and special staining and physiological investigations have accumulated an imposing array of data in support of the view that bacteria contain nuclei and exhibit sexuality. However, this concept is still in controversy. Difficulties in interpretation of morphological observations, even with the electron microscope, are well illustrated in a provocative investigation of the star forms of *Phytomonas tumefaciens,* a bacterial pathogen of plants.[22]

A series of interesting data indicating sexuality in bacteria (*Escherichia coli*) was made by combining the use of ultraviolet irradiation to produce double synthetic deficiencies in two strains; followed by an opportunity for cell fusion of the two strains. The deficient strains were cultivated together in a medium supplying the required pairs of deficient metabolite for each: biotin and methionine for one, and threonine and proline for the other. From the mixture were isolated strains capable of synthesizing *all four metabolites* ("prototrophic" strains), suggesting that fusion between the two sorts of deficient cells had occurred in the mixed culture, with recombination of characters. The prototrophic types normally occurred too rarely in either irradiated strain to be detected in the deficient strains before growth together. While such data are highly suggestive and stimulating they may be susceptible of other interpretations than sexuality and fusion of cells.

Mutual Influences. For example, as early as 1925 data were published indicating that if one species of organism was cultivated in the presence of another, the first would acquire one or more properties of the second. This was spoken of by Burnet as "entrainement."[25] In one study, a harmless streptococcus from cheese was shown to acquire the property of forming erythrogenic toxin (scarlet fever toxin) when grown in contact with scarlet fever streptococci.[26] In another study the virus of rabbit fibroma was changed into myxoma virus by bringing the former into contact with dead myxoma virus.[27] Later it was found that extracts of dead, smooth, type III pneumococci would induce live, *rough, non-type-specific* forms of type II pneumococci to change into smooth type III pneumococci[28] (see Fig. 94). Similar changes have been induced in encapsulated strains of *E. coli*.[29] In 1944 in an epoch-making study it was found that the substance in the type III pneumococcus extracts responsible for the change of the non-type-specific R II strains to type-specific III strains is a highly polymerized desoxyribonucleic (d-r-n) acid.[30] All of these phenomena could be classed as examples of "entrainement."

Nucleic acids as a class have long been recognized as important constituents of the nucleus. That they are important constituents of genes and are hence involved in heredity is indicated by experiments on the dependence of mutation on certain wave lengths (2650 A° and 2280 A°) of ultraviolet light which are specifically absorbed by nucleic acids, nuclear proteins and certain enzymes.[34] These wave lengths are also those most effective in producing mutations in fungi, yeasts, fruit flies and bacteria. Preliminary experiments indicate that the actual substance changed in these experiments is thymonucleic acid. Thus it may be that genes are not essential to bacterial heredity.

The basic importance of these discoveries is incalculable. As exemplified by the transformation of pneumococcal types, here is a substance (desoxyribonucleic acid) of definite composition, taken from one cell, applied to the exterior of another of a different type, which changes the type of the second cell into that of the first, *permanently*. The chemistry of the altered cell is such that it goes on manufacturing more of the mutagenic substance (d-r-n-acid) which in turn maintains the new type specificity. In other words, we have an heredity-changing agent which can be extracted from dead cells and which then becomes part of a new cell—a "transmissible mutagen" or a chemical gene. This, basically, is Lamarckism at the level of the unicellular organism. An analogous phenomenon has been observed as a "killer trait" in *Paramecium*.[36]

This is strongly suggestive of the action of viruses, which become intimately associated with cells and may induce the infected cell to go on manufacturing more of the virus. Indeed, in some virus-induced neoplasms of animals (see Viruses) the *species character* of the virus-infected cell is entirely changed from that of the animal of which it was part. An antiserum against the virus neutralizes, and combines, with the virus and with the cancer cells containing it; but *not with normal cells.* "The malignant animal cell seems to have become completely dependent upon the vital activity of the virus associated with it."[32, 33] The association between the virus and the tissue cell is seen as so intimate and close as to represent a new, hybrid species of cell or a combination analogous to that of the d-r-n in the pneumococcus, which, as it were, "infects" the pneumococcus cell and grows with it.[35]

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Chapter 9

METHODS OF SYSTEMATIC STUDY OF BACTERIA

Variation and Identity. In view of the variability of microorganisms, mentioned in the preceding chapter, it might be thought that the exact identification and description of a species of bacterium would be a useless if not impossible task. This is not the case because variation usually occurs within certain fairly well-known limitations. Practically all forms of life vary to some extent, some more than others. Creatures with which we are thoroughly familiar may vary considerably yet we recognize them without difficulty because (*a*) we are familiar with the variant forms, and (*b*) we recognize certain basic similarities among the variants.

So with microorganisms; although they may at times vary greatly in one or more respects, they seldom do so in all details at once and, normally, under the uniform conditions of laboratory study, they remain quite recognizable as bacteria, and retain fundamental, species-distinguishing characteristics. It is necessary to know what these are, and how they are usually determined in the laboratory. For purposes of discussion let us proceed as though we have been given an "unknown" organism for complete identification and systematic study.

The first important step in the systematic study of a bacterium is to be sure that it really belongs to the class Schizomycetes. From descriptions given earlier in this book there should be no difficulty in identifying an unknown organism as a bacterium and determining that it is not a protozoan, a yeast, a mold, a virus, a rickettsia, an alga or a pleuropneumonia organism. The main differentiating characters may be tabulated for convenience as in Table III.

Purification of Culture. The next step in an exact study of any bacterium is to separate it from other forms with which it might be confused, or which might introduce error into various biochemical experiments or tests of pathogenicity, pigmentation, etc., performed in the course of the identification. This process is spoken of as *isolation in pure culture*. Many a student* has fallen into difficulties by assuming the purity of a culture and neglecting this very important step. Microscopic examination of a smear stained by Gram's method may sometimes reveal the presence of contaminants and very often gives a valuable clue as to the genus or family of the organism but cannot be

---

* As well as veteran researchers!

TABLE III

CHARACTERISTICS OF MICROORGANISMS

Protozoa	*Algae*	*Molds and Yeasts*	*Bacteria*	*Rickettsiae*	*Pleuropneumonia Organisms*	*Viruses*
1. Grow in dark; some on lifeless media.	1. Require sunlight; grow on lifeless media.	1. Grow in dark on lifeless media.	1. Grow in dark on lifeless media.	1. Grow only in living cells.	1. Grow in dark on lifeless media.	1. Grow only in living cells.
2. Multiply by fission and sexual means.	2. Multiply by fission and sexually.	2. Multiply by budding, conidia, and sexually.	2. Multiply by fission only.	2. Method of multiplication unknown. Probably fission.	2. Complex life cycle.	2. Method of multiplication unknown.
3. Much larger than bacteria; in volume usually hundreds to thousands of times greater. Do not pass filters.	3. Much larger than bacteria. Do not pass filters.	3. Size much greater than bacteria — filaments and other structures often macroscopic. Do not pass filters.	3. Round forms do not exceed about 5 microns, usually not over 2 to 3 microns in diameter; rod forms usually not over 2 microns in diameter and 10 microns in length. Some species of spirochetes may exceed 500 microns in length but not over 1 to 2 microns diameter. Do not pass filters (except spirochetes).	3. Much smaller than bacteria. Have rod, coccus, and spiral forms. Some pass bacterial filters.	3. Very minute, except spherical "large bodies". Pass bacterial filters.	3. Not visible by ordinary means. By electron microscope some appear to have a spherical, cuboidal or "tadpole" form. Pass bacterial filters.
4. Internal organs; nucleus; vacuoles "mouth" opening, etc., like higher animals.	4. Cell structure like higher plants.	4. Cell structure, especially nuclei, visible.	4. No definite organs in the cell visible with ordinary microscopes. Granules, spores, capsules.	4. No internal structures visible.	4. No definite internal structure.	4. No definite structures visible.
5. Can ingest solid partcles except a few parasitic forms.	5. Cannot ingest solid particles.	5. Cannot ingest solid particles.	5. Cannot ingest solid particles.	5. Mode of nutrition unknown; presumably like bacteria.	5. Mode of nutrition unknown; presumably like bacteria.	5. Mode of nutrition unknown.

in the absence of sunlight, algae are eliminated. The size and shap of the organisms will show whether or not they are molds, yeast protozoa, or pleuropneumonia-like organisms. They cannot be viruse or rickettsiae since these do not grow in lifeless media. At this poir we may conclude that our organism is a bacterium. If there is sti doubt, appropriate tests may be made along the lines indicated i the table.

An inspection of the growth gives an idea as to the size, shape, colo and consistency of colonies. Let us say that the colonies are about

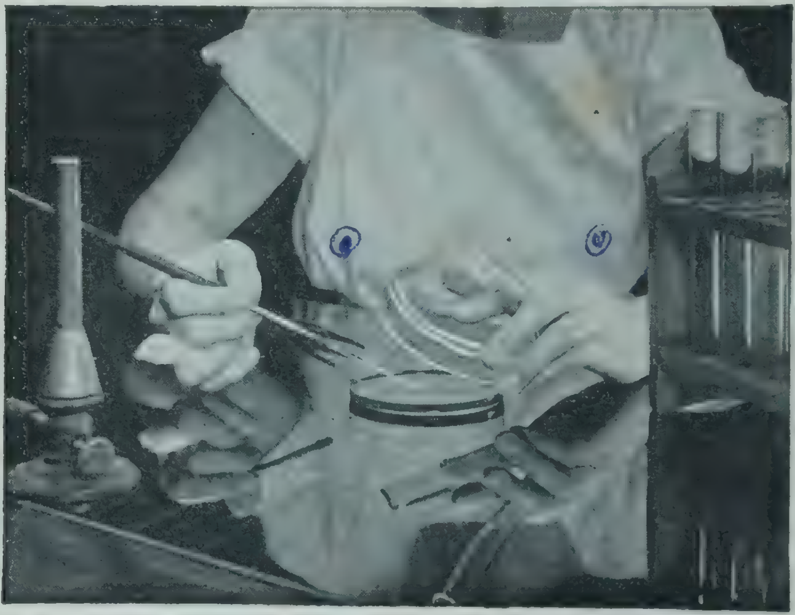

Fig. 98. Subculturing bacteria. Transfer of bacterial colonies from Petri dish to tubes containing culture media. By this method the desired strains of bacteria are isolated. (Courtesy Parke, Davis & Co.)

to 2 mm. in diameter, glistening, convex, circular, opaque, butyrous (butter-like) in consistency and lemon yellow in color.* A convenient way of recording these facts is to use one of the charts published by the Society of American Bacteriologists.[1]

By carefully transferring a portion of one of these colonies (Fig. 98), with a sterile needle into a tube of extract broth (Fig. 99), we provide ourselves with a purified culture, which may be studied further as described below.

If no growth occurred on any of the plates inoculated with the original material, we may assume that:

(*a*) No living bacteria were present in the inoculum; or

**Potato Medium.* Pigment is often beautifully shown on cubes of potato sterilized with a few drops of water in the bottom of a tube.

Name of organism...Source..
Date of isolation...Habitat...
Is phase variation observed?..........................Phase on this Chart: S, R, M, G (smooth, rough, mucoi

Underscore required terms.

Sketches

Vegetative Cells: Medium used...
Reaction (pH)....................Temp........................Age..........d
Size of Majority..
Ends, *rounded, truncate, concave, tapering*

Motility: In broth................................ On agar.............................

Sporangia and Endospores: *present, absent.*
Medium used................pH...........Temp...........Age...........d
Endospore Form: *spherical, ellipsoid, cylindrical*

Irregular Forms:
Present on...................................in...........days at..............°C.

Agar Colonies: Temperature...........°C. Age.......d.
Form, *punctiform (i. e. under 1 m.m. diam.), circular (i. e. over 1 m.m. diam.), filamentous, irregular, rhizoid.*
Surface, *smooth, rough, concentrically ringed, radiately ridged.*
Edge, *entire, undulate, lobate, erose, filamentous, curled.*
Elevation of growth, *effuse, flat, raised, convex.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*

Surface Colonies

Gelatin Colonies: Temperature...........°C. Age.......d.
Form, *punctiform, circular, irregular, filamentous.*
Elevation, *flat, raised, convex, pulvinate, crateriform (liquefying).*
Edge, *entire, undulate, lobate, erose, filamentous, curled.*
Liquefaction, *cup, saucer, spreading.*
Surface, *smooth, contoured, rugose.*
Optical Characters, *opaque, translucent, opalescent, iridescent.*

Surface Colonies

Agar Stroke: Temperature...........°C. Age.......d.
Growth, *scanty, moderate, abundant, none.*
Form of growth, *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
Lustre, *glistening, dull.*
Chromogenesis.......................................*photogenic, fluorescent.*
Odor, *absent, decided, resembling*...
Consistency, *butyrous, viscid, membranous, brittle.*
Medium, *grayed, browned, reddened, blued, greened, unchanged.*

....
Medium:
Temperature
....................°C.
Age
..........d.

Nutrient Broth: Temperature...........°C. Age.......d.
Surface growth, *ring, pellicle, flocculent, membranous, none.*
Clouding, *slight, moderate, strong, transient, persistent, none, fluid turbid, granular growth.*
Odor, *absent, decided, resembling*...
Sediment, *compact, flocculent, granular, flaky, viscid.*
Amount of sediment, *abundant, scanty, none.*

....
Medium:
Temperature
....................°C.
Age
..........d.

Gelatin Stab: Temperature...........°C. Age.......d.
Growth, *uniform, best at top, best at bottom.*
Line of puncture, *filiform, beaded, papillate, villous, arborescent.*
Liquefaction, *none, crateriform, infundibuliform, napiform, saccate, stratiform:* begins in...........d. complete in...........d.
Degree of liquefaction in..............days.......................................
Method used...
Medium, *fluorescent, browned, unchanged.*

....
Medium:
Temperature
....................°C.
Age
..........d.

FERMENTATION

	Monosaccharides							Disaccharides						Polysaccharides						Alcohols					
Medium................. containing................. ....and:	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Melibiose	Cellobiose	Raffinose	Melezitose	Starch	Inulin	Dextrin	Glycogen	Glycerol	Erythritol	Arabitol	Adonitol	Mannitol	Sorbitol
Gas in................................ fermentation tube																									
Amt. CO₂ in Eldredge tube																									
Reaction (pH) after..............d.																									
Titrable acidity in ml. of N/........Na OH																									

TARY DATA

ACTION ON ERYTHROCYTES

Cells: ....
Method: *plate, broth, filtrate*
Hemolysis: *negative, positive*
Methemoglobin: *negative, positive*

PRODUCTION OF INDOLE

Medium ....
Test used ....
Indole *absent, present in* .... *days*

PRODUCTION OF HYDROGEN SULFIDE

Medium ....
Test used ....
H_2S *absent, present in* .... *days*

ACTION ON NITRATES

Medium .... Temp .... °C.
Nitrite: ......d. ......; ......d. ......; ......d. ......; ......d. ......
Gas (N_2): ......d. ......; ......d. ......; ......d. ......; ......d. ......

Medium .... Temp .... °C.
Nitrite: ......d. ......; ......d. ......; ......d. ......; ......d. ......
Gas (N_2): ......d. ......; ......d. ......; ......d. ......; ......d. ......

Ammonia production (in amino-N-free nitrate medium): *negative, positive*
Complete disappearance of nitrate in .... medium: *negative, positive*
Disappearance of 2 p.p.m. nitrite in .... medium: *negative, positive*

REDUCTION OF INDICATORS

Medium .... pH .... Temp .... °C.

Indicator	Conc.	Reduction:
....	..........%	......hr. ......; ......hr. ......
....	..........%	......hr. ......; ......hr. ......
....	..........%	......hr. ......; ......hr. ......
....	..........%	......hr. ......; ......hr. ......

STAINING REACTIONS

Gram: ......d. ......; ......d. ......; ......d. ......; ......d. ......
Method ....
Spores: Method ....
Capsules: Method ....
Medium ....
Flagella: Method ....
Special Stains: ....
....
....

ADDITIONAL TESTS

Methyl red: *negative, positive*
Voges-Proskauer: *negative, positive*
Growth in sodium citrate: *absent, present*
Growth in uric acid: *absent, present*
Hydrolysis of starch: *complete (iodine colorless); partial (iodine reddish-brown); none (iodine blue)*
Nitrogen obtained from the following compounds: ....
....
....
....

SPECIAL TESTS

ERICAN BACTERIOLOGISTS, Dec. 28, 1934, by the Committee on Bacteriological Technic.
isting of M. W. Jennison and H. J. Conn.

(*b*) The temperatures used were not suitable; or
(*c*) Some other medium, possibly with a different reaction (*p*H), is necessary; or
(*d*) The bacteria may have been strict anaerobes.

Suitable adjustments of conditions must then be made until growth is obtained. Sometimes this is very difficult.

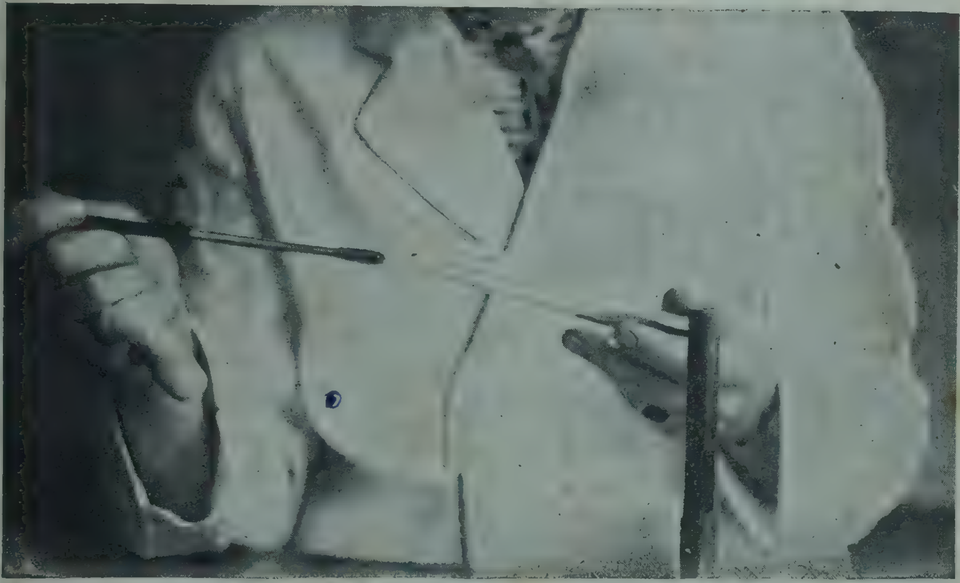

Fig. 99. Manipulation of culture tubes, cotton plugs and inoculating needle. The needle, held as shown, is first heated to redness in the Bunsen flame. The two tubes are held almost horizontally in the left hand, the first (nearest the reader) supported by the index finger, and the second by the middle finger, the base of each tube lightly grasped by the thumb. In this way, the operator has an unobstructed view of the contents of the tubes.

The first plug is grasped and removed by the little finger of the right hand, the second plug is grasped and removed by the fourth finger.

After a few seconds are allowed for the needle to cool, it is quickly inserted into one tube, withdrawn and used to inoculate the other. The plugs are then quickly re-inserted in their respective tubes, the needle flamed and put on the desk. Only then are the tubes placed in their rack. If bits of cotton adhere to the mouths of the tubes after withdrawal of the plugs, they may be singed in the flame momentarily. This process does *not* sterilize the mouths of the tubes. It does no more than warm them. Waving the open tubes to and from the flame is not necessary and introduces dust into them. It is, however, a practice generally indulged in.

Assuming that good growth was obtained on the plates and in the "pure culture," the next step in identification is a study of motility, arrangement, morphology and staining reaction. The first three characters may be determined by observation in a hanging-drop preparation of the growth in broth. The hanging drop has already been described (see page 40). Let us assume that our organism is a coccus (spherical or nearly so), although this must be confirmed by m croscopical examination of a fixed, stained preparation.

Arrangement and Motility. A hanging drop prepared with a *youn broth culture* is a useful means of determining how bacteria are arranged and their motility. Chains of cocci or bacilli are readily observed while cubical packets of *Sarcina* can be seen turning over and over lik little bundles floating in the medium. *Micrococcus* groups appear to b very irregular, like bunches of grapes but may often appear in pairs Motility, if present, is easily seen in hanging drops. Let us assume that our unknown is nonmotile.

Staining Reaction and Morphology. After examining a hanging drop the next step is to confirm the morphology and observe the staining reaction. Gram's stain is of great value, as it not only makes the study of morphology and arrangement more exact but shows us whether we are dealing with one of the great groups of gram-positive or gram-negative organisms. One must bear in mind that Gram's method of staining requires experience and that the differentiations it gives are not absolute. Some organisms are gram-positive only when young or when cultivated on blood or serum media. Let us assume that the organism under discussion is gram-positive.

Other morphological features may be looked for at this point. If the organism is rod-shaped it may produce spores. This is best determined by staining an old (at least one week) agar-slant culture with methylene blue or by a method of spore staining (see page 85). A surer test is to emulsify some of the growth in 2 ml. of sterile water and heat it to 85° C. for ten minutes, afterward inoculating some of the heated material into broth and incubating for several days. If growth occurs, it is practically certain that spores were present.

Capsules may or may not be visible, depending largely on the culture medium. Sometimes they are seen only on organisms in pathological material. They may be determined by capsule stains or darkfield methods. Let us say our organism shows no demonstrable capsules.

The morphology of any organism always varies to some extent. A given variety of bacteria may, in any one culture, produce cells of varying size just as, in a group of men or horses, some may be large and some small. If the bacteria are cocci, some of the individual cells may not be perfectly round and some may be oval. If bacilli, some may be long and thin, others short, oval and thick; some may occur singly, others in pairs or chains or long filaments. But the predominating form, size and arrangement of the cells in a pure culture are usually quite apparent and constitute a most important and reliable differential character. Let us assume that, confirming the indications seen in the hanging drop, the smear shows our organism to be a coccus and to have an irregular, grape-bunch-like type of arrangement.* It seems, therefore, on the basis of the table of classification on page 67, to be a *Micrococcus.*

Summarizing our knowledge at this point, we may state that we are dealing with a nonmotile, gram-positive, unencapsulated coccus, growing well in irregular clusters on plain extract medium as well as blood-infusion medium, at a *p*H of about 7.4, producing opaque, glistening, lemon-yellow colonies and preferring a temperature of about 37° C.

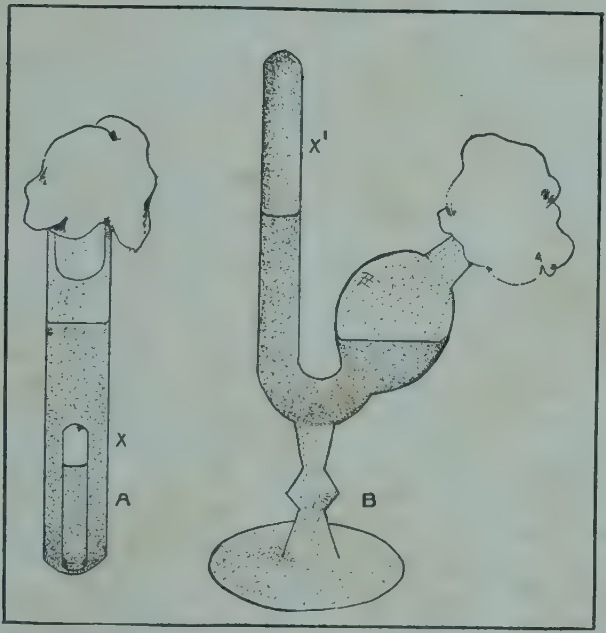

Fig. 100. Types of apparatus used for collecting gas produced by bacteria, *A*, Durham tube (gas has collected at *X*). *B*, Smith tube (gas has collected at *X'*).

There is still, however, a good deal to learn about our unknown before identification is complete.

Biochemical Tests. The experienced bacteriologist would know at once exactly what peculiarities to look for in dealing with a culture of gram-positive cocci which have the characters we have enumerated. For the present, however, we shall put ourselves in the place of a person to whom a gram-positive coccus is a new and hitherto unknown plant.

Since certain enzymes are highly characteristic of bacteria, the power of our unknown organism to ferment or hydrolyze some of the carbohydrates commonly in use, such as dextrose (glucose), saccharose (cane sugar), and lactose (milk sugar), should be tested, as well as its power to hydrolyze or attack such familiar nitrogenous a protein substances as sodium nitrate, sodium nitrite, gelatin, coag lated serum, the casein in milk, etc.

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* Often, in young cultures, the bunches are still small and consist mostly of pairs of newly divided cells. It requires some experience to make the necessary differentiations between the different sorts of coccal arrangements.

In any of these tests it is important that the medium and cond tions of incubation support *good vigorous* growth of the organis A negative result in the absence of normal, good growth is obvious of no value.

Fermentation Tests. These tests may be made as described o page 145. When dealing with an unknown, each tube of broth shoul contain a small inverted vial (placed there *before* sterilization) (se Fig. 100) to catch any gas that may be formed. Gas might otherwis pass off into the atmosphere and not be detected. Two organism both of which ferment the same carbohydrate, may be sharply di ferentiated if one forms gas while the other does not.

Some Fermentable Substances Used in Microbiology

- *Monosaccharides*
  - Pentoses:
    - l-arabinose
    - xylose
    - rhamnose
  - Hexoses:
    - dextrose
    - d-mannose
    - galactose
    - d-levulose
- *Disaccharides*
  - sucrose
  - lactose
  - trehalose
  - cellobiose
- *Trisaccharides*
  - raffinose
  - melezitose
- *Polysaccharides*
  - inulin
  - starch
  - glycogen
- *Alcohols*
  - Trihydric:
    - glycerol
  - Tetrahydric:
    - d,l-erythritol
  - Pentahydric:
    - adonitol
  - Hexahydric:
    - d-manitol
    - dulcitol
    - sorbitol
- *Glucosides*
  - salicin
  - esculin
  - arbutin
  - phloridzin
  - convallamarin
  - colocynthin
  - amygdalin
  - α-methyl-glucoside
- *Organic esters*
  - sodium hippurate
  - sodium citrate
  - sodium formate
  - sodium succinate
  - sodium tartrate
- *Other substances*
  - inositol
  - perseit
  - d-glucosamine (HCl)

When gas is produced from carbohydrates by bacteria, it is *prim facie* evidence of fermentation and is always accompanied by sourin or acid formation since the gas is derived from one of the acids pro duced. However, fermentation often occurs without gas productio and then acid formation is our only evidence that the organism h metabolized the carbohydrate. Sometimes only alkaline substances are produced from carbohydrates, *e.g.,* genus *Brucella.* Acid may be detected by adding to the medium an indicator or dye such as bromcresol purple which changes color in the presence of acid. The change in color of the indicator is our proof of fermentation. However, as has been pointed out, some species can metabolize these acids, so that observations should be made every twenty-four hours in order that the culture may not revert to an alkaline reaction before the acid formation has been noted. Variant strains of the same species may differ considerably in the *rate* at which these reactions are brought about and ample time for observation must therefore be allowed.

Substances frequently used in studying the fermentative powers of various organisms are listed on page 190.

Gases Produced by Bacteria. The nature of the gas formed by bacteria may be of great differential value. Two gases commonly given off by bacteria are carbon dioxide and hydrogen in various proportions. It is often of importance, especially in sanitary bacteriology, to determine the ratio of carbon dioxide to hydrogen. The differentiation is easily made by adding strong NaOH solution to the fermentation tube. This absorbs the CO_2, leaving H. This is described more fully in the section dealing with the microbiology of water (page 584).

Carbon dioxide and hydrogen are not the only gases produced by bacteria, however. Many organisms, in their metabolism of proteins or protein-digestion products, *e.g.,* cystine, taurine and other sulfur compounds, set free hydrogen sulfide, often in large amounts. This gas is one of the most noticeable in connection with putrefactive processes. Some organisms may be identified or differentiated from others by their power to produce H_2S. Thus, *Salmonella schottmülleri,* one of the causes of gastro-enteritis, produces hydrogen sulfide in its decomposition of amino acids, while *Salmonella paratyphi,* a similar species, does not. The test therefore has diagnostic value.

Hydrogen sulfide is readily detected in cultures by the use of lead acetate which reacts with H_2S to form black lead sulfide. The acetate may be added directly to the culture medium, or it may be suspended above the medium in the tubes on strips of sterile filter paper impregnated with the salt, held between the glass and the cotton plug. Iron salts may be used in the same manner.

Methane is another gaseous product of bacterial metabolism. It results usually from the fermentation of cellulose under anaerobic conditions. In swampy places anaerobic bacteria attack the cellulose of dead vegetation and sometimes large amounts of the gas are given off. The bubbles seen arising during the summer time in woodland swamps are nearly always methane, although hydrogen and hydrogen sulfide may also be present in small amounts, as well as carbon dioxide.

Clostridium butyricum, a saprophyte of the soil, and similar organisn
are active in this respect.

Some organisms may produce poisonous gases under certain ci
cumstances. For example, *Pseudomonas aeruginosa* is said to produc
hydrogen cyanide (HCN), while a number of species of *Coryneba*
terium, especially *C. diphtheriae,* produce hydrogen telluride whe
cultivated, as is frequently done, upon medium containing potassiu
tellurite. Many bacteria, especially saprophytic species, also produc
large amounts of ammonia and nitrogen during protein decompositio

Proteolysis. In addition to the above tests, tubes containing commo
proteins (nutrient gelatin, coagulated serum and sterile milk) may b
inoculated to determine the ability of our unknown to attack differen
proteins. These may be incubated with the carbohydrate tubes.

Gelatin. A tube of solidified nutrient gelatin* is inoculated b
"stabbing" a wire, having the desired bacteria upon it, down into th
depths. Many workers prefer to incubate the gelatin at 20° C. rathe
than at body temperature. At the former temperature the gelatin re
mains solid except where digested by the organism, and the shape o
the portion liquefied may be observed. This was formerly regarde
as of great significance, but is really of much less value than othe
tests. The time lost in waiting for growth to occur at 20° C. (if indee
it occurs at all) is much more valuable than the information as to th
form of the liquefied area. Gelatin cultures held at 37° C. liquef
completely due to the temperature but may be placed daily in th
refrigerator for a sufficient time to allow them to solidify if undigested
This should be continued for two weeks unless evidence of digestio
is obtained sooner. An uninoculated tube of gelatin is incubated witl
the others to serve as a guide in refrigeration time. Those tubes i
which the gelatin fails to solidify may be marked "+" or "digested.
The tubes should not be shaken while warm, as growth, and a smal
amount of gelatin digestion, may at times occur *only in the surfac*
layer and this would be masked were it mixed with the bulk of th
warm, fluid culture.

Serum Digestion. Coagulated serum may be prepared by mixin
three parts of horse or beef serum with one part of nutrient broth
Glucose is sometimes incorporated in a concentration of 0.25 pe
cent. This is Löffler's medium so commonly used in the study an
diagnosis of diphtheria. The reaction needs no adjustment if the serun
is fresh. Old serum may have a *p*H of 8.2 or above. Five milliliter
amounts of the mixture are placed in tubes which are then put ir
the autoclave in a slanting position and heated at 100° C. or mor
for an hour. The serum is coagulated and sterilized at the sam
time. The slants are inoculated when cool, by smearing a loopful o
broth culture or growth from agar over their surfaces.

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* Ordinary extract or infusion broth containing 10 per cent gelatin.

When attacked by bacteria the serum usually becomes brownish and translucent and the growth appears to sink inward. Total liquefaction sometimes occurs in forty-eight hours at 37° C., especially in cultures of aerobic spore-forming bacilli, but is often delayed for as long as two weeks.

Action on Milk. Milk is rich in nutrient substances: casein, fat, mineral salts, lactose, vitamins and other materials. It has a *p*H of around 6.8 when fresh. It is an ideal culture medium for many bacteria, especially those adapted to growth in body fluids, *i.e.,* parasites like *Salmonella typhi* and *Corynebacterium diphtheriae.* Its sanitary relationships thus become clear.

For use as a bacteriological culture medium, skimmed milk is tubed in 5 ml. amounts and sterilized by autoclaving. If an indicator, such as litmus or bromcresol purple, be added, fermentation of the lactose may be detected. In addition, rennet production may be inferred if the milk is curdled (provided this is not due to souring; a point difficult to determine if fermentation of the lactose also occurs). Digestion of the casein often follows coagulation. The milk then becomes brownish and translucent and the clot disappears.

Another method of testing the ability of many organisms to hydrolyze test substances like serum or starch is to mix the test substance with nutrient agar, pour into Petri plates and, when solid, heavily inoculate the surface. After good growth has occurred a reagent, reacting with the test substance to produce some distinctive appearance, is flooded over the surface of the agar. If hydrolytic enzymes have been produced the colonies will be surrounded by zones where no reaction occurs. For example, gelatin or serum may be added to the agar. By flooding the plate (after incubation) with several ml. of a solution consisting of H_2O, 100 ml., $HgCl_2$, 15 gm., HCl, 20 ml., unhydrolyzed gelatin or serum will be coagulated to a white opaque appearance. The colonies of hydrolyzing organisms will be surrounded by a clear zone.[2] Starch plates may be made similarly, and treated with Lugol's iodine solution. The starch hydrolyzing colonies will be surrounded by colorless zones. The remainder of the plate will turn dark blue.

However, these plate methods do not measure the *degree* of hydrolysis. Starch, for example, may be split by diastatic action so that it does not produce a blue color, yet no acid may be produced.

Other Proteins. In addition to casein, gelatin and serum, other protein substances such as cooked egg and ground-up beef are sometimes used for studying bacteria. In fact, one may use any protein his ingenuity suggests. In all tests upon proteins and protein-decomposition, carbohydrate-free media should be used as acids produced from fermented carbohydrates interfere with protein hydrolysis.

Fibrin. Tillett and Garner[3] have developed a method of testing certain specific proteolytic activities of pathogenic streptococci. These organisms, especially the human pathogens, possess the power of digesting the fibrin clot of human blood in a short time.* They can also digest human tissues.[4]

The production of ammonia by the decomposition of amino compounds and from other biochemical reactions is of great value in certain bacterial differentiations. This may be detected by inserting a moistened strip of red litmus paper between the cotton plug and glass tube of a culture so that ammonia fumes arising from the culture will affect the litmus. The litmus may also be affected by volatile amines, so that the test is not wholly accurate.

Bacterial Reductions. In addition to tests with carbohydrates and proteins, other features of the physiology of the organism may be studied. A physiological property characteristic of many bacteria is the power to reduce various compounds.

Nitrate Reduction. One method of determining the power of reduction is to incubate the bacteria being investigated in broth containing about 0.1 per cent of sodium *nitrate* ($NaNO_3$). After forty-eight hours and at other intervals, a test is made for the presence of *nitrites* ($NaNO_2$) by withdrawing a little of the culture from the *bottom* of the tube and immediately adding a drop of sulfanilic acid solution† and a drop of dimethyl-alpha-naphthylamine solution‡ or by allowing the drops to settle to the bottom of the whole culture.

The development of a red color denotes the presence of *nitrites*, but the failure of this color to develop raises a question. Either (*a*) the nitrate has not been attacked, so that no nitrite is present or (*b*) nitrite

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* Fibrinolysin test (*Tillett and Garner*):

1. In a test tube place 0.2 ml. of fresh (not over 48 hours old) oxalated human plasma. (Oxalated plasma is prepared by drawing 10 ml. of blood into a tube containing 0.02 gm. of sodium oxalate, or 1 ml. of 2 per cent oxalate. Centrifuge and use the clear supernatant fluid. Keep cold.)
2. Add, successively:
   - (*a*) Normal saline solution, 0.8 ml.
   - (*b*) Broth culture to be tested (24-hour-old, well-grown culture is necessary), 0.5 ml.
   - (*c*) $CaCl_2$ (0.25 per cent in 0.85 per cent NaCl), 0.25 ml.
3. Mix and immediately place in water bath at 37° C.
4. Observe the time of solid coagulation, and then measure the interval required for the clot to be liquefied. This may vary from 15 minutes to several hours.

† *Sulfanilic acid solution:*

Glacial acetic acid	100.0 ml.
Water	250.0 ml.
Sulfanilic acid	2.8 gm.

‡ *Dimethyl-alpha-naphthylamine solution* (*Wallace and Neave*[5]):

Glacial acetic acid	100.0 ml.
Water	250.0 ml.
Dimethyl-alpha-naphthylamine	2.1 ml.

has been formed but also attacked and reduced to free nitrogen or ammonia. A test to see whether any nitrate remains may be made by adding a little pulverized zinc. This reduces any remaining nitrate to nitrite (which may be tested for as above) and tells us whether or not the organism has utilized *all* of the nitrate. If this test is positive (*i.e.,* nitrate is still present), the original test for nitrites having been negative, then it is clear that the organism did not attack the nitrate at all. If both the nitrate and nitrite tests are negative then it is obvious that the organism utilized all the nitrate as well as nitrite.

Nitrite Reduction. The latter point, *i.e.,* ability of the organism to attack nitrite, may be determined separately by testing the ability of the organism to destroy the nitrite in cultures known to contain it. This is done by incubating cultures containing quantities of nitrite so small (about 0.002 per cent) as just barely to give a positive test for nitrites. If the organism is capable of reducing nitrite, the culture will soon lose its power of reacting positively to the nitrite reagent, because the organism will quickly reduce all of such a small amount of nitrite.[6] A sterile control tube should be tested at the same time, as illuminating-gas fumes often contain nitrous acid which may be absorbed by the medium and give a slight reaction.

Reduction of Litmus. The ability of an organism to reduce other substances than nitrates and nitrites is often investigated. Litmus, for example, is often used as an acid-indicator in milk cultures, but it also serves to show whether or not the organism has strong reducing powers by becoming entirely decolorized when reduced. Just enough is added to the medium before sterilization to give a definite color.

The Reductase Test. Methylene blue is similarly decolorized by many organisms. The dye is used as a hydrogen acceptor in respiration (see section on bacterial respiration, page 292). Many other compounds are similarly utilized. Standardized solutions of methylene blue are often added to milk samples to estimate, roughly, whether a few, a moderate number, or enormous numbers of bacteria are present. When great numbers are present, the blue color may disappear almost immediately; when less, it may remain unchanged for six hours or more. Such a test is known as a methylene blue reduction test or, since the reduction was thought to be due to an enzyme called reductase, the *reductase test.* The rate of reduction also depends on the *kind* of bacteria present, other hydrogen acceptors present in the solution, availability of oxygen, etc. (see section on microbiology of milk, page 636).

Indol. Indol is a substance resulting from the attack of bacteria upon the amino acid *tryptophane* (see section on metabolism, page 302). It is usually tested for in cultures made in broth prepared with peptone containing tryptophane. Cultures are incubated for forty-eight to seventy-two hours. Indol reacts with acidified solution of Ehrlich's reagent (para-dimethyl-amido-benzaldehyde)* to produce a pink compound.

The culture to be tested should be shaken with 1 ml. of xylol. Indol is soluble in xylol and is concentrated in it and carried to the surface by the solvent after a minute of standing. A few ml. of Ehrlich's reagent are then *gently* added to the culture and are made to remain in a layer between the xylol and the medium. If indol is present a pink color forms in a few minutes as a "ring" at the junction of the xylol and the reagent. Pink compounds *not* soluble in xylol sometimes develop in the presence of Ehrlich's reagent if the xylol is not added first. These are not due to indol.

There are many other tests which are used in microbiology; some only for very specific purposes in diagnostic work; others in special phases of research. A few are described in other parts of this book, and others in the literature.[1, 7]

Identification of the Unknown Organism. Test media prepared as above described, are inoculated from the purified culture of our "unknown" and are incubated as stated (37° C.) and carefully observed until all changes have ceased and all tests have been completed. An incubation of four to five days is usually sufficient. A tabulation is then made of the results. For the organism under investigation, let us assume that they are as follows:

Pigment (as observed in colonies on agar)—lemon yellow.
Lactose—fermented; no gas.
Dextrose—fermented; no gas.
Saccharose—fermented; no gas.
Salicin—not fermented.
Gelatin—slowly liquefied.
Agar slant and cubes of potato—good growth, soft, moist and glistening, lemon yellow in color.
Broth—turbid, sediment is yellow; faint pellicle (scum).
Milk—coagulated, acidified.
Nitrates—reduced to nitrites.
Indol—not produced.

How are we now to determine the genus and species of the gram-positive, irregularly arranged cocci which we have been studying?

Use of Keys. Although every experienced bacteriologist has at his finger tips, so to speak, all of the distinguishing cultural reactions and other identifying characters of the organisms with which he is working, it is unusual, to say the least, to find one who knows *all* the char-

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* *Ehrlich's reagent:*

Ethyl alcohol (95%)	380 ml.
HCl (conc.)	80 ml.
Para-dimethyl-amino-benzaldehyde	4 gm.

acters of *all* the species. When an unknown organism is encountered which must be identified, the main morphological and tinctorial features are determined in some such manner as just described and then recourse is had to *keys* or other reference works. Probably the most useful key for general bacteriological use is "Bergey's Manual of Determinative Bacteriology."[8, *]

Genus and Type Species. There are certain central types of bacteria as, for example, *Streptococcus pyogenes, Bacillus subtilis, Clostridium butyricum,* and the like. Each of these is a well-known, thoroughly studied, easily identifiable species representative of a genus or a group of species, and is spoken of as the *type species* of that genus or group of organisms. Usually it is the first-described member of the species. To every experienced bacteriologist each of these names conveys a very definite idea as to the characters of the group, although illy defined, partly studied organisms frequently distinguishable from the type species or from each other only with the greatest difficulty or not at all or, on the other hand, differing so markedly from the type species that the relationship seems very vague, are often included in the group.

In order to use "Bergey's Manual" we should first determine to which of the five orders of the class Schizomycetes our culture belongs. On pages 65 and 66 of the manual is to be found a brief synopsis of the characters used to differentiate the five orders and suborders. Obviously the species in question is neither spiral, elongate or flexuous, filamentous, sheathed or characterized by iron or sulfur deposits (Orders III, IV, and V). Neither is it branching (Order II). It does not possess photosynthetic pigment nor form long stalks, hence does not belong in suborders II or III of Order I. We are thus left to consider Suborder I, the Eubacteriineae.

On pages 67 and 68, we find a synoptic description of the families of the Suborder Eubacteriineae. Our organism is spherical and may therefore fall into either family I (Nitrobacteriaceae), family V (Micrococcaceae), family VI (Neisseriaceae) or family VII (Lactobacteriaceae). It is obviously not a member of the other families, which include only rod-shaped or filamentous bacteria. To settle the choice between families I, V, VI, and VII, more exact information is necessary and we turn to page 69. On page 69 *et seq.,* we find that in only one genus of family I, the *Nitrosococcus,* are the organisms spherical. But the organisms of this genus do not grow on ordinary heterotrophic culture media, whereas our "unknown" does, and will not grow autotrophically. This, therefore, leaves us to consider families V, VI, and VII and to them we turn our attention.

Reference to page 295 makes it clear that we are not dealing with Neisseriaceae (family VI) since they are gram-negative; and we elimi-

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[*] All names of bacteria given in this book are based on the 1948 edition of "Bergey's Manual" unless otherwise noted.

nate at present also the Lactobacteriaceae (page 305, tribe Streptococceae) since our organisms grow well on ordinary extract agar without blood and do not occur predominantly in chains but in irregular clumps and masses as well as in pairs.

Family V (Micrococcaceae), therefore, would seem to be our objective, and we are referred to page 235. Here we find that the descriptions of genera II and III (*Gaffkya* and *Sarcina*) do not correspond with the organism in question, and that we must search in genus I. The organisms of this genus resemble each other so closely that it is necessary to consider carefully the characters of the individual species.

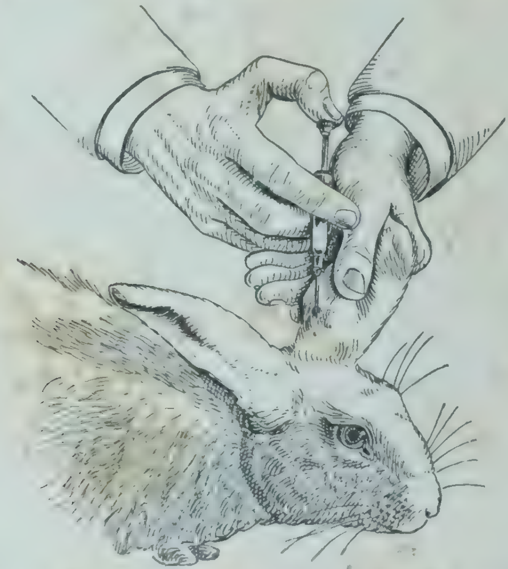

Fig. 101. Injection of bacteria into a rabbit through a vein in the ear. (McFarland.)

A short study of the data we have already obtained by our cultural tests shows that our organism corresponds closely with the description of *Micrococcus citreus* (page 242), since it produces a lemon-yellow pigment, liquefies gelatin, ferments lactose, grows on potato with lemon-yellow pigment, reduces nitrates to nitrites and fails to produce indol.

A further check upon the identity of the culture may be made by testing other fermentable substances and comparing various characters of organisms closely resembling it, such as *Micrococcus flavus*. A few repetitions of the tests usually serve to confirm the diagnosis, or prove it to be in error, necessitating further study.

Pathogenicity may be determined by injecting 0.5 ml. of a twenty-four-hour broth culture intravenously and subcutaneously into a rabbit or guinea pig (Figs. 101 and 102). If our unknown is truly pathogenic, abscesses will probably form or the animal may die. Some strains are more pathogenic than others. Further details concerning micrococci will be given when these genera are taken up specifically later (see page 535).

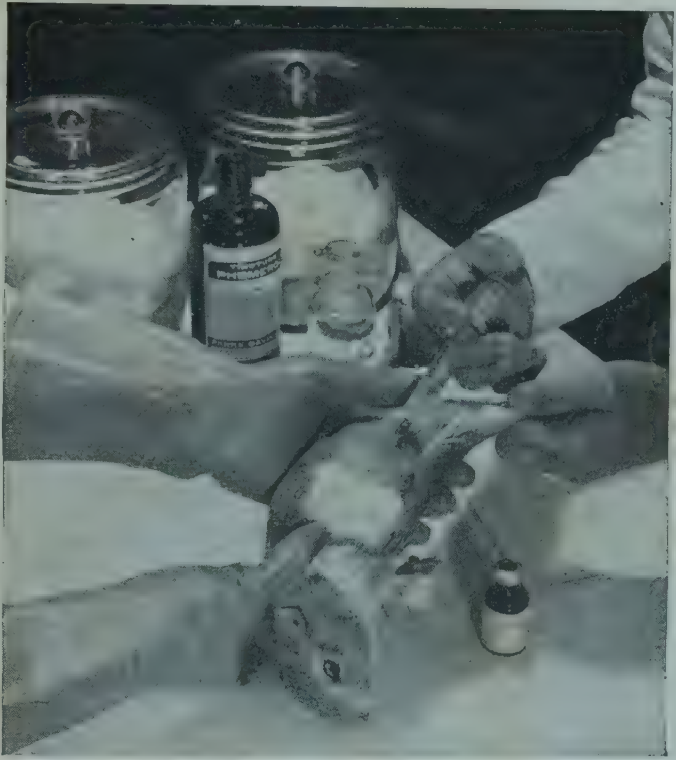

Fig. 102. Method of inoculating a guinea pig with microorganisms: this injection is into the peritoneal cavity. (Therapeutic Notes, October, 1942, Parke, Davis & Company.)

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Chapter 10

STERILIZATION AND DISINFECTION

1. Uses of Heat and Filtration

Purposes and Definitions. There are four main reasons for killing or removing microorganisms. They are: (1) to prevent infection of men, animals and plants; (2) to prevent spoilage of food and other commodities; (3) to prevent their interference in various industrial processes depending on pure cultures; (4) in research, in order that studies of the growth of one kind of organism in a particular medium or infected animal may not be confused by the presence and growth of others at the same time.

Several substances and procedures are involved in accomplishing these results. These may be defined as follows:

Sterilization. Sterilization means the freeing of any object or substance from all life of any kind. This is accomplished usually by heat but sometimes, in special cases, by the use of chemicals, ultraviolet irradiation or filtration.

Bactericide. Any substance or agent killing bacteria is a bactericide or bactericidal agent. The suffix, -cide, indicates "killer" and is used with germ, virus, etc.

Disinfection. Disinfection, as the word implies, means the killing or removal only of organisms capable of causing *infection* and does not necessarily require that *all* organisms be killed. However, many processes of disinfection actually accomplish sterilization. Disinfection is usually accomplished by chemicals like carbolic acid, formaldehyde, iodine or bichloride of mercury. In the case of milk, disinfection, *but not sterilization,* is brought about by *pasteurization,* a heating process to be described.

A disinfectant is an agent accomplishing disinfection. The term is often used synonymously with *antiseptic*.

Sepsis is the growth of harmful microorganisms in living tissue.

Asepsis. In a strict sense, asepsis is the practice of keeping *infectious* microorganisms away, but the term is usually applied to any technic designed to keep *all* microorganisms out of the field of work or observation. The work of a microbiologist and of a surgeon involves aseptic technic. The surgeon and his assistants have sterile instruments, handle them with sterilized gloves, cover the patient with sterilized sheets except for such area as is necessarily uncovered, and wear sterilized caps, gowns and masks to prevent infected dust, droplets of saliva, perspiration or sputum from entering the sterile field and possibly infecting the patient. The patient's skin cannot be absolutely *sterilized* without injury, but the site of the operation is *disinfected* as thoroughly as possible by applications of iodine or some other suitable disinfectant.

In microbiology the worker uses sterilized culture media and sterilized glassware kept sterile, until the moment of use, by coverings of paper and cotton plugs and by *aseptic technic.*

Mechanisms Involved. The microbial cell, like all living cells, depends for its life and normal functioning in great part on the maintenance of its protoplasm or cell substance and its enzymes in a properly reactive condition. This, in turn, depends on a continuance of its normal chemical composition, its respiratory enzymes, metabolism, etc., and the maintenance of its colloids (see page 79) in a normal physiological state. When any of these factors is disturbed, growth is interfered with and life may cease if the disturbance is sufficiently severe or prolonged. Advantage has been taken of these points of vulnerability of bacteria, etc., to perfect methods of killing them or inhibiting their growth.

One type of method is directed primarily toward alteration of the *chemical composition* of the protoplasm, or of some particular vital constituent thereof, for example, a respiratory enzyme. Another type consists in destroying the *colloidal state* of the protoplasm. Some methods in common use accomplish both chemical and physical change; the action of others is not wholly understood. We shall first discuss methods of producing physical changes in the protoplasm. These affect mainly its colloidal state, although various little-known chemical changes also doubtless occur.

Coagulation. Protoplasm, as we have seen, is a typical colloidal substance, consisting largely of particles of proteinaceous matter (the so-called discontinuous or disperse phase) suspended in an aqueous menstruum, the cell fluid (continuous phase). Colloidal particles suspended in near-neutral aqueous fluids possess negative electrical charges which cause the particles to repel each other and help keep the particles in suspension.

In most metallic colloids, such as colloidal gold, the boundary of the particles is sharply defined and the transition from the gold to the water phase is sudden and distinct. There is no hazy, ill-defined zone around the gold particle where the aqueous phase and the gold phase are intermingled. Such metallic colloidal particles are easily thrown out of suspension and separated from the water if their negative charges are neutralized—for example, by the presence of

H^+ ions or Na^+ ions. Their colloidal state is very unstable and they are classed as *lyophobic* ("solution-fearing") colloids.

Protoplasm particles, on the other hand, are much more stable colloids because the boundary between particle and aqueous phase is indistinct. The material of the protoplasmic particles is less dense and much more highly hydrated at the surface, and the degree of hydration increases from within outward until, at the outermost surface, it may be difficult to determine exactly where the disperse phase ends and the aqueous phase begins. This state gives such colloids great stability and they are classed as *lyophilic* ("solution-loving") colloids. Many organic colloids, as gelatin, agar, globulin, mucin, etc., are of this nature. However, if sufficient H^+ be added, lyophilic colloids may be precipitated (curdled or coagulated) by neutralization of their surface charge.

Lyophilic colloids such as protoplasm, gelatin, etc., may be made to imbibe, or combine with, or enmesh, or engulf (depending on the colloid) the aqueous phase by chemical treatment and also by temperature changes. The whole mass may then become solid. Gelatin and agar remain fluid when kept hot, but solidify or "set" on cooling. In contrast, protoplasm, egg white, globulin, albumin, etc., remain fluid at physiological temperatures but become solid and apparently dried out when heated to around 80° to 90° C. This is called coagulation and is familiar to anyone who has boiled an egg or heated blood or serum. The general rule may be stated that any agent inducing coagulation is lethal to living cells.

It is seen from the above that two general types of agent may cause coagulation (resulting in death of protoplasm): (*a*) heat and (*b*) cations, *e.g.*, H^+ or other positively charged particles.

Of the coagulative agents other than heat, several are in common use as disinfectant substances. Some of these are heavy metal salts. Of metallic ions it may be said that in general coagulating power is exponentially related to valency and atomic weight; the trivalent, heavy metals having much greater than one-third more coagulative power than bivalent metals. It is also *important to note* that in concentrations below those necessary to produce coagulation some heavy metal salts and other disinfectants exert noxious effects because of *specific poisoning action* on some cell constituent. In the concentrations in which some disinfectant heavy metal salts (*e.g.*, $HgCl_2$) are commonly used, this may be the more important action. In still lower concentrations they may stimulate growth.

Bichloride of mercury is one of a number of substances which coagulate protein rapidly in high concentrations, and that is one reason why it is a good disinfectant. It also has more complex actions of reversible, physicochemical nature related to the sulphhydryl group.

This will be discussed later. Phosphotungstic acid, carbolic acid (phenol), formaldehyde, alcohol, copper sulfate and salts of a number of other heavy metals also coagulate protein more or less readily and all, therefore, have a microbicidal and cytotoxic action. These agents as well as other antimicrobial substances will be discussed in the next chapter. Here we will give attention to the effects of heat and methods of sterilization by mechanical means.

The Role of Hydration in Protein Coagulation. The role of *hydration* or water content in protein coagulation is an extremely important one and is clearly shown by experiments on the effect of heat on egg albumen under various degrees of hydration. The results of some of these experiments are shown in the following data:[1]

Egg albumen + 50 per cent water coagulates at...... 56° C.
Egg albumen + 25 per cent water coagulates at...... 74°–80° C.
Egg albumen + 18 per cent water coagulates at...... 80°–90° C.
(boiling water = 100° C.)
Egg albumen + 6 per cent water coagulates at...... 145° C.
Egg albumen + 0 water coagulates at.............160°–170° C.
(oven temperature)

Obviously coagulation proceeds best where the protein is well hydrated. The less the degree of hydration, the more resistant is the protein to coagulation. The same principle holds true in coagulation by chemicals. The resistance of bacterial spores to heat and chemical disinfectants may be explained as being due either to the dehydrated condition of the protein or protoplasm within them or to the fact that the free water of the vegetative protoplasm, before spore formation, became closely united or *bound* with the protoplasmic material when the spore was formed. In either case the water no longer functions as a separate aqueous phase. Examples of the resistance of dehydrated protein to the action of coagulative agents are seen in the difficulty encountered in making dried-egg powder coagulate. Heated in a test tube over a naked Bunsen flame, it will turn brown or char, but not unless a considerable quantity of water has been added will it coagulate. Of course, mixing dry egg with dry chemical disinfectants such as powdered bichloride accomplishes nothing at all till moisture is added. The same is true of dried-milk powder. It would be difficult indeed to make cottage cheese without plenty of water.

For these reasons *aqueous* solutions of disinfectants which coagulate are generally more useful than solutions of the same substances in solvents other than water. For example, alcohol, a mild coagulating agent, is more effective in 70 per cent aqueous solution than in pure, absolute alcohol containing no water, since it hydrates the protein to be coagulated.

STERILIZATION BY HEAT

Heat may be applied for sterilization in three ways: (*a*) by steam or hot water (moist heat); (*b*) by prolonged baking in the oven (dry heat); and (*c*) by complete incineration. The last needs no comment beyond pointing out that (1) common sense will direct what may be burned up and what should not; and (2) care must be taken to see that the material is completely burned.

Factors in Heat Sterilization. At the temperatures commonly used for sterilization (100° C. to 170° C.) several factors affect the rate at which microorganisms are killed. Important among these are the hydration, the temperature, the time of exposure, and the *p*H.

Time and Temperature. Obviously these are inversely related. The lower the temperature, as a rule, the longer the time required to kill the organisms. At 120° C. in an autoclave (compressed steam), for example, it may require only five or six minutes to kill everything, even very resistant spores; whereas at 100° C. in the same autoclave the same spores may survive six hours or more. Even increasing the temperature from 100° C. to 105° C. may diminish the killing time to two hours.

Numbers of Organisms. As will be seen more in detail in the section on disinfection with chemical agents, sterilization is an orderly, continuous, process in which there is a definite relationship between initial numbers of organisms, the efficacy of the lethal process, and the elapsed time. In other words, under ideal conditions, for any uniformly acting lethal agent the number of organisms remaining alive after a given interval is a function of elapsed time. This is shown below.

SURVIVORS OF 100° C. AFTER VARIOUS INTERVALS

Elapsed time (minutes)	Surviving organisms (per ml.)
0	90 million
5	12 "
7	8 "
9	5 "
11	3 "
15	1 "
20	200 thousand
25	20 "
30	0

If these relations be drawn on a chart we have something like the following, which is a representative form of curve for many lethal agents. This shows that the majority of cells are killed quickly but that some may survive for a considerable time, depending on lethality of the agent, hydration, *p*H, osmotic pressure, age of cells, etc.

Other Factors. We have already indicated the relation of *hydration* to killing. In addition, heat increases dissociation of H^+ *ions* (*p*H) and the effectiveness of (H^+) in disinfection processes. Fluids of high *osmotic pressure* tend to dehydrate the cell contents and so increase heat resistance. The *age of the organisms* is also important, older cells being more heat resistant than young ones. The *species of organism* is a determining factor; the form of the survival curve varying markedly with different kinds of organism exposed to the same lethal influence.

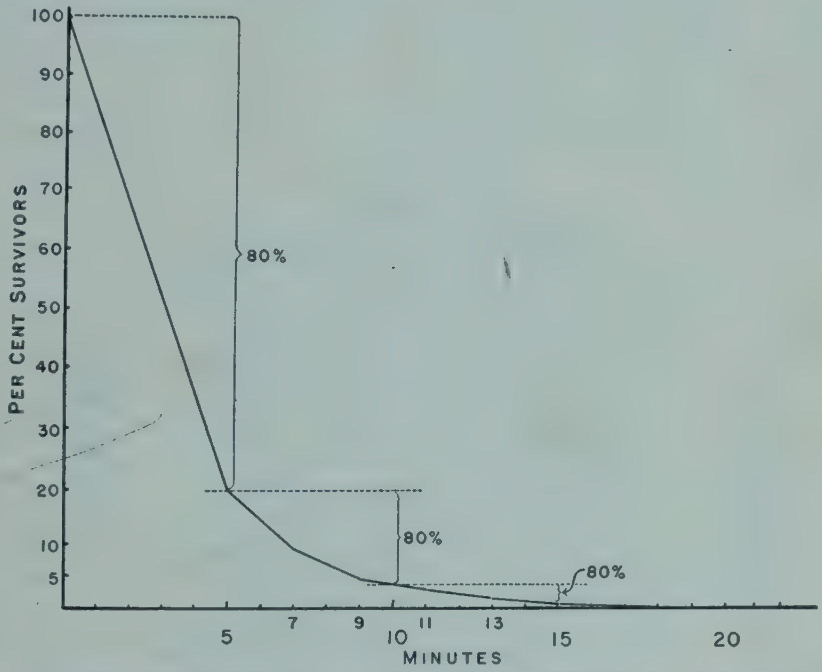

Fig. 103. Relation between time and death of organisms in a situation in which the lethal agent acts at a constant rate under uniform conditions.

The close relation of *p*H and temperature in lethal processes is illustrated by the following:

Relation of *p*H and Temperature to Survival of Tetanus Spores[2]

*p*H	Survival (minutes) at			
	105° C.	100° C.	95° C.	90° C.
1.2	4	5	6	6
4.1	6	11	14	23
6.1	9	14	38	54
7.2	11	29	53	65
10.2	5	11	21	24

Here it is seen that (OH^{+}) is also an adjuvant of heat, but to a lesser degree than (H^{+}). Acidity and alkalinity both increase the lethal effect of heat.

Boiling in Water. The use of hot water for sterilization and disinfection is very simple. It is only necessary to remember that spores may remain alive even after an hour of boiling. For ordinary household purposes of *disinfection*, but not *sterilization*, five minutes' boiling of dishes, clothes, etc., is usually sufficient, provided the hot water actually comes into contact with the microorganisms, and not merely with the outside of lumps or packets or other objects containing them.

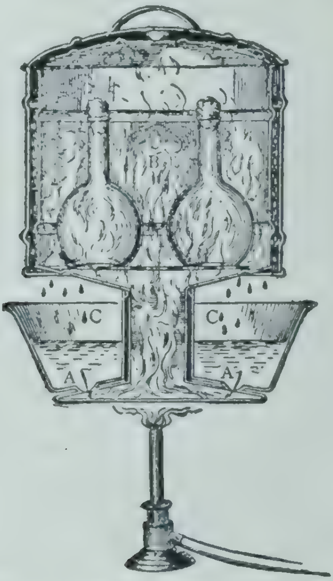

Fig. 104. Arnold sterilizer used for tyndallization. Water from *C* passes through holes (*A*), comes into contact with the heated bottom of the vessel and rises as steam into the chamber *B*, where it condenses and drips back into *C*.

In *all* disinfection and sterilization, *contact* between the microorganisms and the agent used is absolutely essential. Boiling can never be depended on *absolutely for sterilization*, especially at high elevations above sea level.

Free-flowing ("Live") Steam. "Live" or free steam is usually applied in an apparatus like that shown in Figure 104. Boiling water and free steam never reach a temperature above 100° C. (212° F.). In mountainous regions it is much lower than 100° C., as at Tahoe, California, elevation about 10,000 feet, where boiling occurs at about 94° C. Free steam is sometimes used to accomplish *fractional sterilization* or *tyndallization.*

Tyndallization. Tyndall, a famous British scientist, noticed that a period of about eighteen to twenty-four hours' incubation at ordinary room temperatures is usually sufficient to enable dormant and heat-resistant spores to germinate and pass into the vegetative state in which they are sensitive to heat. He devised a process of sterilization based on these observations, which consists in steaming for a few minutes at 100° C. on three or four successive occasions separated by eighteen- to twenty-four-hour intervals of incubation at favorable temperature. The intervals permit the dormant spores to become vegetative. This process renders an infusion sterile whereas one single, continuous boiling for one hour may not, since a few of the spores remain in their dormant and resistant state during this time. This intermittent process is not frequently used today in the laboratory but is put to practical use in the home canning of goods. A great disadvantage of the method is the time required to achieve complete sterilization. An advantage is that it requires no special apparatus. In some fluids, as water, spores may not germinate promptly. Also, if the material is freely exposed to air, anaerobe spores may not germinate and may survive the process.

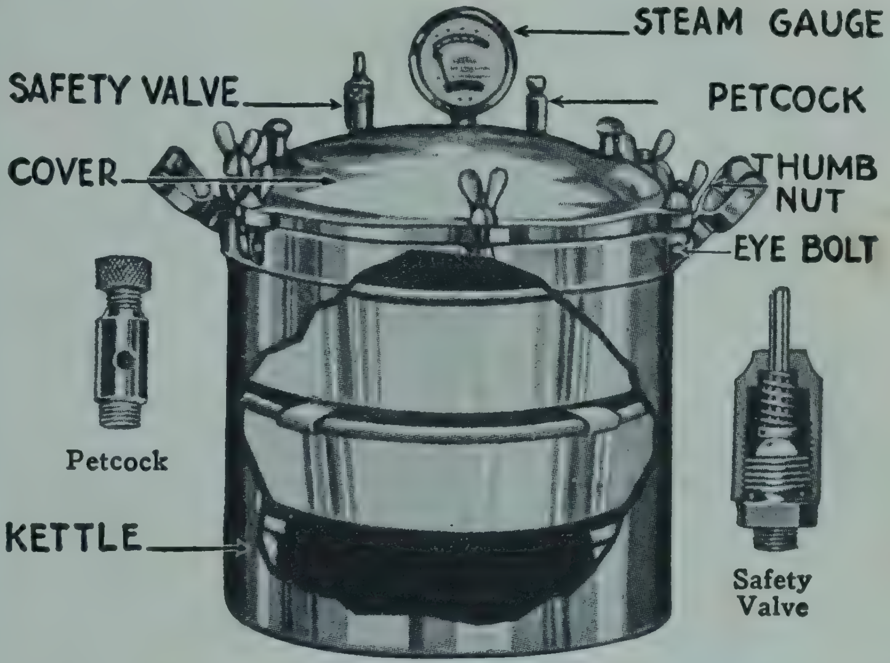

Fig. 105. A modern type of pressure cooker. About 1 quart of water is placed in the bottom of the kettle. When it boils, steam forces air out through the petcock which is left open till pure steam emerges. When it is closed, pressure is registered on the steam gauge and, if the flame is not lowered, will escape through the safety valve. The thumb nuts and eyebolts are used to tighten and hold the cover. Details of the petcock and safety valve are shown in the insets.

Compressed Steam. Autoclaving. Anyone familiar with the operation of a pressure cooker (Fig. 105) is familiar with an *autoclave* because the cooker is a simple form of autoclave. In the autoclave

(Figs. 106 and 107), be it a small and simple home pressure cooker or an apparatus large enough to fill a room and fitted with various

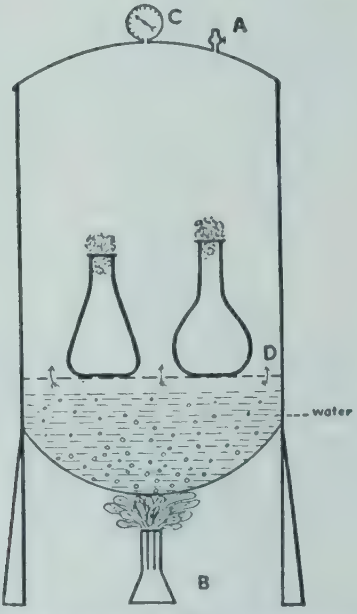

Fig. 106. Diagram showing structure of a pressure cooker or simple form of autoclave. At *A* is shown the valve used to allow the escape of air. *C* is a combined pressure gauge and thermometer. *D* is a shelf, perforated to allow the steam to rise from below. (Park and Williams, "Pathogenic Microorganisms," Lea and Febiger, publishers.)

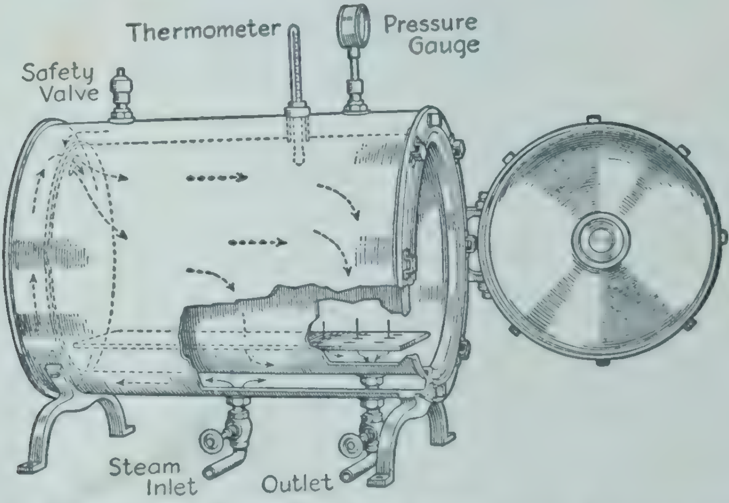

Fig. 107. Autoclave of modern double-jacketed type, showing pathways of steam and essential parts. (Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company, publishers.)

gauges, pipes, valves, clocks and wheels, the object is to heat the articles to be sterilized by means of steam under considerable pressure.

Steam under pressure is hotter than boiling water or free-flowing steam such as is used in tyndallization. The higher the steam pressure, the higher the temperature.

The relation of *steam* pressure to temperature is shown in the following table:

Temperature °C. (at sea level)	Pressure (*Pure* steam)
100	0
109	5
115	10
118	12
121	15
124	18
126	20

It must be remembered, however, that it is the compressed *steam* (moisture, *hydration*) that sterilizes and not compressed air (dry and usually not as hot as steam).

For example, as shown by Underwood, pure steam at 15 pounds pressure has a temperature of 120° C. If the steam is mixed with an equal amount of air, at the same pressure the temperature is only 110° C. while if the mixture is 2/3 air, the temperature is only 109° C.

Steam *hydrates* and thus promotes coagulation. Air does neither. Steam, being water vapor, also produces *hydrolysis* at autoclave temperatures. Dry air cannot do this at any pressure or temperature. In autoclaving, therefore, as in using a pressure cooker, a valve is left open for the escape of all air *before the steam pressure is allowed to rise* (Fig. 106 *A*).

The actual amount of water present as steam in an autoclave is small and articles soon dry off after removal, especially if removed from the autoclave while hot.

The *thermometer* on the autoclave is the important guide to the process of autoclaving and not the *pressure* gauge, although the latter, as well as a steam-escape or safety valve, is desirable for *safety*.

The common practice in autoclaving fluids or freely exposed surfaces such as those of dishes and instruments is to apply 115° to 125° C. (10 to 20 pounds' pressure) for twenty minutes. The pressure must be allowed to subside *slowly* after the heating is over or *superheated* fluids in the vessels will boil over. Any large, solid masses must be heated a longer time, to allow for heat penetration. Packages should be spaced so as to allow free circulation of steam. Substances like mineral oil or petrolatum, sand, etc., or any dry objects in tight jars, or which are *impervious to moisture,* cannot be satisfactorily sterilized in the autoclave. The *temperature* may rise as high as 125° C., but, in the absence of moisture, this is ineffective. Such materials are more effectively sterilized in an oven.

Dry Heat. Dry heat is used in oven sterilization (Fig. 108). It is necessary to bear in mind that moist heat, at 100° C. or above, kills bacteria mainly by coagulating their protein just as hot water coagulates an egg, and that only thoroughly hydrated protein coagulates readily. Coagulation does not occur when moisture is not present.

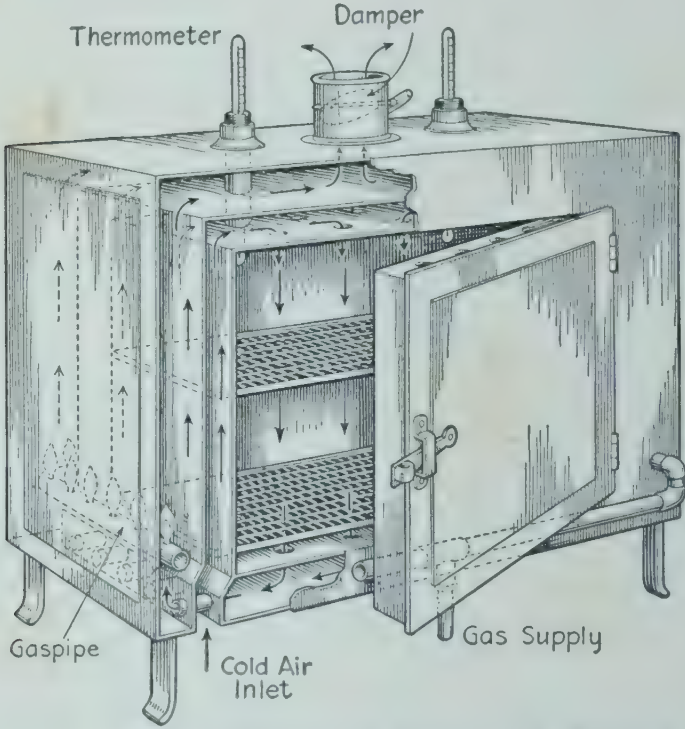

Fig. 108. Hot-air sterilizer. (Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company, publishers.)

Articles in ovens are very dry and therefore, in order to be freed of live spores, must reach a very high temperature (165° to 170° C.; 329° to 338° F.). It is customary to apply 165° C. for a period of two to three hours. This accomplishes not only coagulation, but, what is more effective, slight charring.

A home oven can easily be used for sterilization. A moderate temperature (330° F.) is satisfactory, and the process should be allowed to proceed for three hours. Paper wrappings should be browned but not brittle; muslin or string should be yellow or light brown, due to the heat.

Only dry articles not injured by baking (glassware, bandages, instruments, mineral oils, petrolatum, and the like) may be thus sterilized. Solutions containing water, alcohol or other volatile substances will, of course, boil away and be ruined.

Thorough Heating Necessary. In any process of disinfection or sterilization by heat it is *absolutely essential* that the object be heated through and through and that the *center* of the object be held at a killing temperature long enough to destroy the bacteria.

Thus, in canning, a quart jar of spinach may be held in free steam (100° C.) for five or ten minutes and when grasped with the hand will feel very hot. However, large masses of nonfluid materials like quart jars of canned vegetables and roast meat, in which the contents cannot *circulate,* require a long time (1½ to 2 hours), even in the autoclave, to be heated so thoroughly that the center reaches a bactericidal temperature. Pieces of meat or vegetables to be sterilized in jars by heating should be loosely packed, allowing space for circulation of the fluid in the jar. Air pockets in the jars should be carefully removed. Penetration of the heat is facilitated if the cans or jars or pieces of roasting meat, etc., be small and not packed too closely together, facilitating free circulation of steam or hot air around and between them.

STERILIZATION BY FILTRATION

An exceedingly useful method of freeing fluids from bacteria is by means of filtration. This is seen in its most primitive form when polluted water, seeping through the earth, reappears as spring water, crystal clear, ice-cold and often sterile or with very few bacteria. The bacteria and impurities are removed by passage through the soil provided it is not heavily polluted. This principle is used in purifying city water supplies by passing the water through beds of sand (see Chapter 34). In the laboratory the same principle is used on a smaller scale, and the procedure is modified to suit conditions and accomplish a more certain removal of bacteria. Specially graded and purified clays, fine unglazed porcelain, kieselguhr and the like take the place of the earth in the natural process. For certain filters (Seitz, etc.) compressed paper discs take the place of clay. The clays or porcelain are shaped into convenient, hollow cylindrical forms, closed at one end, and mounted on funnel-shaped stems. They are sterilized by heat before use, so that bacteria in the filter or glassware will not contaminate the filtrate (filtered product). In addition, whereas gravity is the principal force which makes ground water pass downward through the earth, the laboratory process makes use of atmospheric pressure or the centrifuge to supplement gravity. A discussion of the physics of filtration is given in the section on viruses (see page 795).

Various forms of laboratory filter are used, a very common one being shown in Figure 109, and another in Figure 110. A very convenient filter, using compressed paper discs as the filtering agent, i the Seitz type (Figure 111). The Swinney syringe filter is the adaptation of a minute Seitz type filter to fit on the end of a hypodermi syringe.

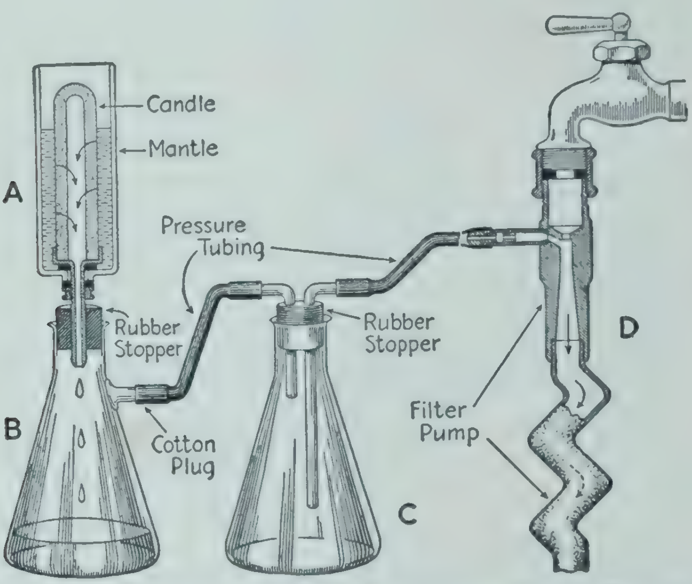

Fig. 109. Typical filtration apparatus. The hollow "candle" of filtering clay, surrounded by the fluid to be filtered, enclosed in a glass cup or mantle, is seen at *A*. The fluid passes through the clay and is drawn into the flask *B* by suction developed by the pump *D*. The flask *C* is to trap any fluid that might be sucked back from *D* or over from *B*. The cotton plug in the side arm of *B* is to prevent ingress of dust when *B* is disconnected from the tubing. (Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Co., Publishers.)

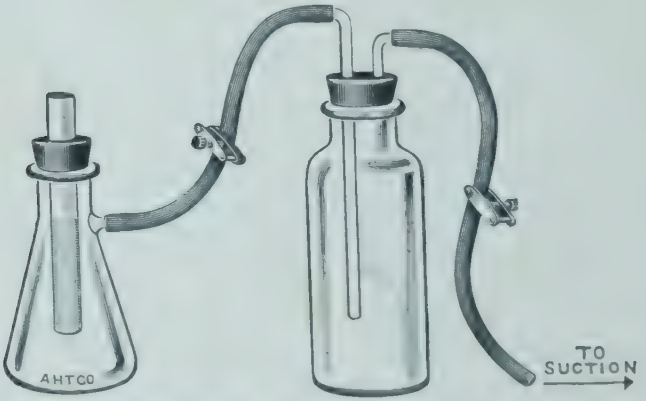

Fig. 110. Porcelain filter of the Chamberland type.

Another adaptation of the Seitz principle is seen in the Boerner centrifugal filter. The base of the filter sets into the mouth of a centrifuge tube which receives the contents of the filter cylinder as centrifugal

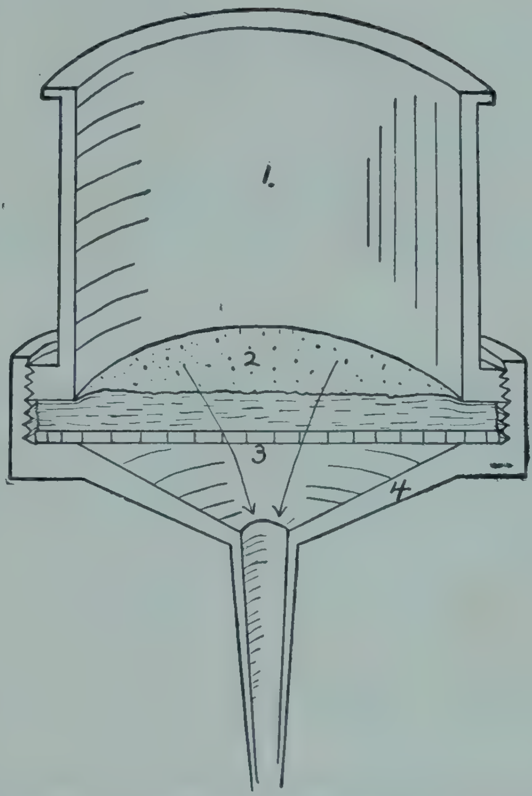

Fig. 111. Seitz type filter. The fluid to be filtered is poured into the cylindrical cup (1). The fluid passes through the bacteria-retaining disc of compressed paper or asbestos (2) which is supported on a wire screen (3). The stem of the funnel-shaped base (4) is inserted in a suction flask through a rubber stopper and the whole assembly sterilized before use. (Morse and Frobisher)

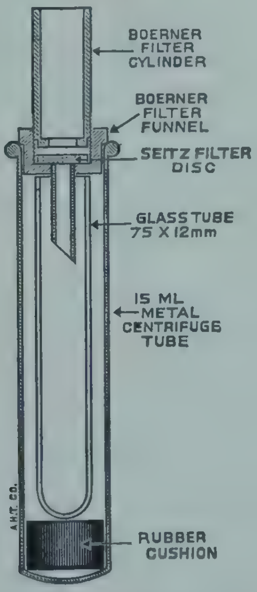
BOERNER FILTER CYLINDER
BOERNER FILTER FUNNEL
SEITZ FILTER DISC
GLASS TUBE 75 X 12mm
15 ML METAL CENTRIFUGE TUBE
RUBBER CUSHION
A.H.T. CO.

Fig. 112. Showing Boerner filter in position in 15 ml. metal centrifuge tube, with glass collecting tube.

force causes the fluid to pass downward through the tiny disc o compressed paper (see Figure 112). An advantage is that suctio devices are not needed, another is that quantities of fluid as sma as 1 ml. may be filtered.

Centrifugation. Microorganisms may be removed from fluids by cen trifugation at very high speeds (15,000 to 30,000 r.p.m.). Such speed multiply the apparent weight of particles by thousands of times the pu of gravity (G). The method is used principally in studies of viruse (Chapter 46).

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Chapter 11

STERILIZATION AND DISINFECTION

2. Uses of Chemicals

Substances which kill, or inhibit the growth of, microorganisms may, for convenience, be classified in three large groups: (1) *disinfectants,* which kill microorganisms within a relatively short time (up to about one hour) after contact;* (2) the *bacteriostatic* agents,* like basic dyes and sulfonamide drugs, which do not immediately kill the cell but inhibit multiplication so that the microorganisms die after hours or days without significant increases in number; (3) antimicrobial agents of biological origin, like penicillin, generally called antibiotics, usually bacteriostatic in action.

Bacteriostatic agents of groups 2 and 3 will be discussed under their appropriate headings later on. Here we may consider the first class of agents, the chemical disinfectants. There are thousands of such chemical agents and each class of substance, and each individual substance, has its own peculiar mode of action which may differ with different kinds of organisms (due to their different physical and chemical structures) and under circumstances involving different temperatures, concentrations, *p*H, presence of other chemical agents, ions, organic matter, colloidal status of the organism, and so on. The physics and chemistry of chemical disinfection are too complex for complete discussion here. We shall merely present the main principles underlying disinfection with chemical agents in general, with special reference to certain disinfectants which illustrate those principles.

Factors Affecting Chemical Disinfection. There are several factors which affect both heat sterilization and chemical disinfection. Of basic importance is contact between the microbicidal agent and the micro organism. In dealing with chemical agents, wetness (low surface tension) is a determining factor. There are two aspects of this facto First, the accumulation (adsorption) of surface-tension-reducing disinfectants on the surfaces of cells; second, the effect of surface tensio reducents on the wetting and spreading properties of the solution.

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* The distinction between a disinfectant and a bacteriostatic agent is entirely arbitrary and depends on what one may call killing "quickly", and inhibiting or, what amounts to the same thing, killing "slowly." One could say that death within a time less than the maximal for one generation under optimal growth conditions (20 minutes to many hours, depending on species) might be regarded as more than mere prevention of multiplication and therefore "true killing or disinfectant" action; while, if death were delayed beyond such time, one might regard death as due only to factors other than the agent applied, acting on cells which have merely been prevented from multiplying. This could represent "true bacteriostasis." There are many obvious difficulties in making such a distinction. The term bacteriostasis is, however, widely used and generally means delayed death, *i.e.,* killing only after some hours or longer.

Contact; Surface Tension. For example, upon adding carbolic aci to a broth culture of bacteria, contact between disinfectant and bacteria is immediate. The bacteria float naked, as it were, and are reache by the disinfectant in effective concentration without delay partly because *carbolic acid lowers surface tension* (see page 131) and is therefore *adsorbed* upon their surfaces. Many disinfectants lower surfac tension and some of them will be referred to later on.

Once adsorption of the surface-active substance on the organism occurs, further action depends on specific toxicity of the anion, cation *p*H, temperature, species of organism, etc.

Wetness. An illustration of the superior spreading properties of a disinfectant solution with low surface tension or wetness is seen in the action of an alcoholic solution of iodine as compared with that of an aqueous one. If crystalline iodine be added to an aqueous solution of potassium iodide, it will dissolve readily and can be brought to the same strength as the alcoholic solutions sold in drug stores as "tincture of iodine." Put a drop of such an aqueous solution of iodine on the leveled back of the hand or on a table top. It remains in a droplet does not tend to spread very far, and does not, unless "rubbed in," come into very intimate or extensive contact with the skin or crevices and pores in the wood. A drop of the alcoholic solution, on the contrary, spreads immediately and appears to soak into, and to wet thoroughly, the material being disinfected. Alcohol is thus seen to be much wetter than water from a physical standpoint (as well as in a politica sense). If organisms be coated with an oily or waxy film, as is the case naturally with tuberculosis bacilli, even when a disinfectant reaches them it affects them with difficulty unless it possesses the special physical property of lowering surface tension, thus allowing it to *wet* the wax.

Conversely, if a surface-tension reducent be added to a *nutrient* solution, it greatly facilitates contact of the nutrient fluid with the organism and stimulates growth. The use of this principle in cultivating tubercle and diphtheria bacill is discussed in the sections dealing with those organisms.

Competitive Adsorption. Soap, in itself a *mild* disinfectant with respect to certain bacteria, has, like alcohol, the power of lowering the surface tension of water and this not only induces the soap to be adsorbed on the surfaces of the bacteria but also permits the water to wet surfaces more thoroughly. Carbolic acid and related compounds, like cresol, have these properties also and are, in and of themselves, powerful germicides besides. A combination of *liquid* soap, carbolic acid and cresols would, therefore, seem to have exceptional possibilities as a disinfectant. Indeed, mixtures containing these substances in *effective proportions* are widely used in hospitals, laboratories, etc. Products of this type are available on the market, or can be made up as *liquor cresolis compositus* from the U. S. Pharmacopeia. An *excess* of soap, however, will interfere with the adsorption of the disinfectants.

In general, the addition of a relatively ineffective, very surface-active substance like soap to disinfectant solutions is likely to be disadvantageous because the soap may be adsorbed to the exclusion of the more effective substance in the mixture.[1, 2]

Likewise, it has been demonstrated by Valko and Dubois[3] that the antibacterial action of certain toxic (disinfectant) surface-active cationic[*] detergents,[†] *e.g.*, soap-like disinfectants may be prevented by the simultaneous use of similar agents of *low toxicity*. Using *Zephiran*[‡] as a highly toxic, surface-active disinfectant, it was found that the toxic action could be prevented or reversed with sodium dodecyl sulfate (Duponol PC), a surface-active disinfectant of relatively *low* toxicity. Staphylococci treated with 1:3000 *Zephiran* could be revived by treatment with the dodecyl sulfate after ten minutes. The reviving action of the *less* toxic substance is presumably due to competitive adsorption—the harmless agent displacing many of the toxic cations from the available spaces on the bacterial surfaces.

Time, Temperature, Concentration and pH or pOH. Disinfectant solutions are not magical. Their values differ in different situations, depending on the kind of bacteria to be destroyed and their location (open wounds, skin, floors, dishes, clothing). No matter what disinfectant is selected for use under any given circumstances, four important factors besides surface tension play a part in the results. These are:

A. *Concentration.* In general, the more concentrated a disinfectant, the more rapid and certain its action.

The effectiveness is, however, not linearly related to concentration, but exponentially. For example, doubling the concentration of phenol in a solution does not merely halve the killing time for bacteria but may reduce it by as much as 98 per cent.

Inhibition and Stimulation. Some interesting observations in this connection appear to be of fundamental significance although exact explanation requires

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[*] In *cationic* disinfectants the toxic or disinfectant ion is an organic *cation*. In *anionic* disinfectants the toxic or disinfectant ion is an organic *anion*.

[†] Detergent: from Latin, *detergere*, to clean.

[‡] A mixture of high-molecular-weight alkyl-dimethyl-benzylammonium chlorides representing a widely used type of modern disinfectant (a typical cationic disinfectant).

further study. It is well known that many substances which are very tox
relatively large amounts actually stimulate growth at certain very low conce
tions. Examples are the action of very dilute solutions of bichloride of me
and lead salts (see page 118). Also, if a bright piece of a heavy metal, as co
silver or gold, be placed on a plate of nutrient agar previously heavily inocul
with an organism such as *Micrococcus aureus*, and the plate incubated, s
quantities of the metal appear to dissolve in the agar and inhibit growth of
organisms in a wide zone around the metal piece. This phenomenon is ca
the _oligodynamic* action_ of metals.

At the periphery of this barren zone one might suppose that, as the concen
tion of metal ions diminishes with distance from the metal piece, growth w
be at first slight, close to the zone, and increase in density to a normal maxim
in all areas where the metal ions had not yet migrated. On the contrary, in m
cases, growth is heaviest at the periphery of the sterile zone, where concentrat
of the toxic ions is minimal. Here a narrow but distinct opaque ring of ex
dense growth is often clearly evident. The same phenomenon is seen in sim
experiments with many other toxic substances.

Hormetic Action. The phenomenon just described *may* play a role in the curi
phenomenon called hormesis.[4] In treating experimentally infected animals w
various drugs, it has been observed that, whereas large adequate doses redu
mortality rates significantly below those of the untreated but infected anima
very minute or therapeutically inadequate doses *increased mortality* rates s
nificantly above that of the untreated controls, *suggesting* that the infecting
ganisms were stimulated in some way.

A similar phenomenon of stimulation instead of inhibition has been dem
strated for trace quantities of streptomycin and penicillin.[5]

B. *Time.* No disinfectant, as ordinarily used, acts instantly. S
ficient time for contact, and for whatever chemical or physical reacti
occurs, must be allowed.

Rate of Disinfectant Action. As in sterilization by heat, chemi
disinfectant action is an orderly, continuous process. Under ideal a
uniform conditions of temperature, concentration of disinfectant, *p*
etc., the rate at which death of the organisms occurs is often const
and the number alive at any moment a function of time only. In su
cases the rate of death proceeds as though the disinfectant w
reacting with molecules of a single substance in the organism un
the laws of mass action—a monomolecular reaction. In such d
infection, if the logs of the numbers of surviving organisms are plot
against units of time a straight line is obtained. If this line is nea
perpendicular obviously the *rate* of killing is high; if it slopes gen
the rate is low; the rate, high or low, is *uniform* throughout and
such cases the *slope* of the line is affected only by such extrane
factors as temperature, *p*H, and concentration of disinfectant.

Unfortunately, disinfectant action is not so simple. The *form* of
curve is dependent to a large extent on the species of organism. T
reaction between disinfectant and organism may proceed as a

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* Action in minute concentrations.

tri-, or quadrimolecular process or one of an even higher or mixed order, suggesting (*a*) that the reactions between protoplasms of different species and different disinfectants are very complex, and (*b*) what appears to be a reaction of a monomolecular type probably is the algebraic sum of a number of simultaneous reactions, the net result of which, by chance, superficially resembles a monomolecular reaction.

C. *Temperature.* As a rule, the warmer a disinfectant, the more effective it is. This is based partly on the principle that chemical reactions in general are speeded up by raising the temperature. As a rule, a rise in temperature of 10° C. increases reaction rates two to eight times. It is particularly true of coagulation by H^+ ions. However, adsorption is decreased as temperature increases. Further, since many disinfectant actions are physical in character, the laws governing chemical reactions do not apply.

D. *pH and pOH.* As a general rule, the lethal or toxic action of harmful agents, both physical and chemical, is affected by (H^+) or (OH^-). The relationship between heat and (H^+) has already been discussed. The increase in effectiveness of benzoates and salicylates in acid solutions, as shown by Rahn and Conn,[6] is an excellent example of the effect of (H^+) on antiseptic action (see chapter on food, p. 658). These authors and others[7] showed that, with the majority of weak acids, the undissociated molecule and not the anion per se is the active agent, and that the effect of increased (H^+) is to suppress ionization of the acid, thus increasing the concentration of the undissociated toxic molecules. Cationic disinfectants* are not favored and may be interfered with to some extent by increased (H^+). On the other hand, increases in (OH^-) favor many of them.

According to Rahn and his associates,[8] practically the only *ions* causing death or stasis of microorganisms (bacteria), are H^+, OH^-, and heavy metal cations. Most disinfectants, both organic and inorganic, appear to depend almost entirely, if not wholly, on the effects of the undissociated molecule.

Inactivation of Antimicrobial Agents. *Nonspecific Organic Matter.* Substances like phenol, strong acids, chlorine, etc., tend to combine with and destroy or coagulate *all* proteins or colloids. The presence of considerable quantities of proteins or colloids such as blood serum, mucus, etc., extraneous to the microorganisms in any material being disinfected, will therefore protect the organisms to a great extent by combining with the disinfectant before it reaches the organisms. Many substances which are *very active* antimicrobial agents in aqueous solution are much less effective, or worthless, in the presence of extraneous materials (*i.e.,* in blood, pus or feces) with which they readily combine.

In the case of antimicrobial agents which act in cells through combination with a *single, specific* molecule or radicle in the cell, su for example, as the sulphhydryl (–SH) group, or a certain part o single enzyme, the presence of non-specific organic matter is less lik to interfere, because it is not rich in free SH groups, much less ri in certain parts of a single enzyme. Such specifically toxic antimic bial agents are represented by penicillin, etc., and, to a lesser e tent, by sulfonamide drugs and some other microbicidal drugs li quinine. Some of these will be mentioned later on.

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* Those in which the effective portion is the cation.

Specific Inactivators. Many antimicrobial agents may be inactivat by the extraneous presence of certain specific substances with whi they react readily. Simple examples are the inactivation of acid by t presence of a carbonate or hydroxide; the precipitation of tox sodium oxalate by calcium chloride. These are specific inactivato Bichloride of mercury, while exerting irreversible coagulative effec in high concentrations (1:1000 or stronger) also exerts a *specific* a *reversible* chemical effect in dilutions of 1:100,000 or more. Filde in 1940, showed that the toxic chemical action of mercury is exert through specific combination with the —SH group in vital portions the cell and that the toxic action may be completely neutralized putting labile —SH compounds such as glutathione and cysteine (s page 298) into the solution. These combine with the $HgCl_2$ before reaches the cells. *Staphylococcus aureus* in contact for seventy-tw hours with 1 per cent $HgCl_2$, and apparently nonviable, may "revived" by treatment with H_2S, which precipitates the Hg as Hg The slow lethal action of phenol on micrococci and on *Salmonel typhi* may be stopped and the apparently dead organisms "revive by removal of the phenol with activated charcoal or ferric chlori Highly reactive thiol compounds (those containing the —SH and r lated groups), inactivate a variety of antimicrobial agents wheth these contain mercury or not. This leads to the general concept th antimicrobial agents which may be so inactivated act on microbes combining in some way (in many cases *reversibly*) with the —S groups in the cell. The position of the —SH groups is usually in vital important enzyme systems so that by their inactivation the vital pro esses of the cells are blocked. The specific differences in effectivene of the various antimicrobial agents with respect to different species organism would depend on whether or not the agents could rea the —SH group and, having reached it, whether the —SH group w in a chemical linkage such that it could react with that particul toxic agent and in a vital situation in the cell. The more general toxic agents, such as $HgCl_2$, would be able to reach and react wi the —SH group in a wide variety of cells; the more specific agent such as penicillin which is toxic for only certain bacteria, would hav a more restricted combining power.[9, 15]

Inactivation of the sulfonamide drugs is discussed with those compounds.

Selection of Disinfectants. While the general principles outlined above will serve as a guide in the selection of disinfectants, *it must be remembered* that because of the chemical and physical peculiarities of each kind of organism, the chemical and physical peculiarities of each disinfectant, and the fact that we do not have *all* of the data as to how each substance acts in each situation, many problems in disinfection are special cases and cannot safely or efficiently be dealt with on the basis of general principles. Full discussion of all these "special cases" would be impossible here. We may, however, consider briefly some representative situations and some commonly used disinfectants.

As to common or representative situations we may classify several as:

(1) the animal body
    (*a*) exterior (skin and mucous surfaces)
    (*b*) interior (gastrointestinal tract; intravenous; intramuscular; etc., sites).
(2) domestic (floors, clothing, eating utensils, etc.)
(3) agriculture (barns, poultry houses, etc.)
(4) wastes (sewage, etc.)

As to common disinfectants we may list:

(*a*) Inorganic substances ($HgCl_2$, Cl, $CaOCl_2$, I, H_2O_2, strong alkalis)
(*b*) Phenol and phenolic compounds (phenol, cresols, resorcinols)
(*c*) Alcohols
(*d*) Quaternary ammonium compounds
(*e*) Soap-like compounds
(*f*) Sulfonamide drugs
(*g*) Antimicrobial agents of biological origin (the so-called antibiotics)

It would greatly simplify matters if we possessed an "ideal" disinfectant; one which could be used in all situations. It would have to possess a number of properties which, unfortunately, are not all found in any single substance, although some of the disinfectants prepared in recent years approach the ideal. This ideal disinfectant should be:

(1) actively microbicidal in high dilutions
(2) not poisonous or irritating to man or lower animals
(3) unaffected by extraneous organic matter (specific in its action)
(4) not corrosive, or able to stain or discolor skin, fabrics, etc.
(5) odorless and tasteless
(6) soluble in water

(7) a good wetting agent (good contact and penetration)
(8) inexpensive
(9) active over long periods (stable)

Since all disinfectants possess one or more of the undesirable pro ties suggested by the list, we must select those having properties able to a given situation and not possessing properties unsuit or dangerous in that situation. For example, a suitable selection general use inside the body might appear to be penicillin. This sesses in reasonable degree properties 2, 3, 4, 5, 6. However, i not a wetting agent, is relatively expensive and unstable. More imp tant, it is highly selective in its action on bacteria, besides which i not actively microbicidal but bacteriostatic and requires the aid of live animal body to be most effective. But its value is unquestion in certain "special cases," when properly applied by a physician. would not use phenol as a substitute, for obvious reasons.

Some Useful Disinfectants. A few substances commonly used as d infectants may be briefly discussed here.

Tincture of iodine is the most generally useful for small cuts a abrasions. If the alcohol causes too much pain, the tincture may diluted with two to four volumes of water. An aqueous solution co taining 1.85 per cent of iodine and 2.2 per cent of potassium iodide excellent but has a high surface tension. Anderson and Mallm have shown the value of colloidal iodine for certain purposes.[16] penetrates well.

Alcohol (70 per cent) alone or containing *not over* 0.01 per ce bichloride of mercury, may be used in similar situations or as a ha wash. If used with $HgCl_2$, it should be followed by soap and wat

Alcohols in general are not very effective as disinfectants. As adjuvant ethyl alcohol is useful because it increases the wetting pro erties of aqueous solutions of other disinfectants. It is frequently us as a hand wash, with small amounts of green soap, perfumes, etc., other disinfectants, mixed in. Its disinfecting power per se is slig for most organisms. Much depends on the chemical structure of t alcohol.[17, 18]

Weak aqueous carbolic acid (phenol) solution of 0.5 to 1.0 p cent has a lower surface tension than water and may be used f washing and dressing in emergencies, but carbolic dressings shou never be left in contact with skin, mucous membrane or wounds f more than thirty minutes to an hour unless a *very* dilute solution used, as they will cause coagulation of the live tissues (gangren which may be followed by serious infection. *Aqueous 1 to 2 per ce crude carbolic* solutions (also *cresol* and similar compounds) mal excellent disinfectants for household use, as well as in the barn.

Cresols form colloidal (milky) suspensions in water and are ther fore especially effective because each colloidal droplet consists *oncentrated* cresol. This is true of any colloidal disinfectant. Their ses are similar to those of phenol and they are more effective and ss expensive. There are many excellent cresol preparations on the arket. They have a clinging odor.

Hydrogen peroxide is an oxidizing agent, breaking down readily to water and liberating oxygen. If it can be prevented from thus ecomposing too rapidly, it is a fairly effective bactericide, but under ost conditions of application its effect is too transitory to be of value ecause of its instability. It is of value in deep dirty wounds as it revents growth of anaerobes one of which causes lock-jaw.

Solution bichloride of mercury (1:10,000 aqueous) is useful for eneral purposes also, but its strong coagulative action must be borne mind. Like phenol, it will cause irritation and corrosion of skin, ucous membranes and wounds if left too long in contact (hence e old name "corrosive sublimate"). *All* mercury compounds are oisonous and should never be used internally. Its action on bacteria ay be reversed by iodine and some other compounds (see page 219).

Within the last few years attempts have been made to decrease he toxic, corrosive and irritating qualities of mercuric disinfectants y incorporating mercury in complex organic compounds. This has elded a number of products which are less toxic and irritating than gCl_2 and may even be used internally *within limits*. Some of these ompounds are highly effective *in vitro;* in the presence of organic atter they are less effective but still far better than $HgCl_2$. Possibly he best known of these compounds are *Mercurochrome* (a red dye olecule incorporating Hg), *Merthiolate* and *Metaphen.* Other excelnt mercurials are phenylmercuric nitrate, very effective and of sufciently low toxicity for limited internal use; and ammoniated mercury, ong used as a 10 per cent ointment for external wounds, fungal infecons of the skin, etc.

Chloride of lime (0.5 to 1 per cent aqueous solution) is excellent or similar purposes and is inexpensive. It depends for its effectiveness n the liberation of free chlorine which, in turn, liberates oxygen rom water. The ordinary chloride of lime of commerce is unstable nd soon loses most of its free chlorine. Many new compounds which etain their chlorine and liberate it more slowly are more effective nd efficient. *Azochloramid* is one of these; *dichloramine toluol* is anther. The odor of chlorine may be objectionable.

The mode of action of the hypochlorites in aqueous solution is not lear. Various theories ascribe the antimicrobial action to HOCl, HCl, "nascent" oxygen and free chlorine. Thus: $2CaOCl_2 + 2HCl \rightarrow CaCl_2 + 2HOCl$; $HOCl + HCl \rightarrow H_2O + Cl_2$; or, $2HOCl \rightarrow 2HCl + O_2$; or, $2Cl + H_2O \rightarrow 2HCl½O_2$; or $Cl_2 + H_2O \rightarrow HCl + HOCl$.

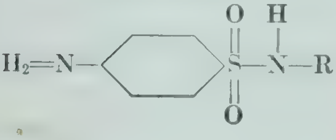
In addition to the above substances, there are many new proprietary chlorine compounds on the market based on various of the principle given above.

New patented chlorine products and dyes, phenol derivatives, etc are designed for various uses. Specific applications and directions fo use generally accompany packages of these proprietary preparation

Sulfonamides. This class of compounds has a brilliant history a therapeutic agents in certain infectious diseases.[19-24]

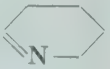
The sulfonamides commonly used today are chemically related a shown below:

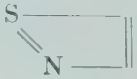
Type structure:

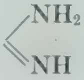


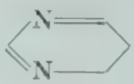
Derivatives

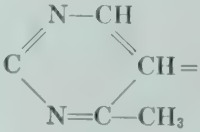
When R is H =sulfonamide

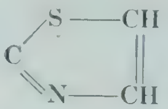
When R is  =sulfapyridine

When R is  =sulfathiazole

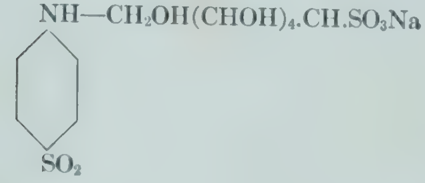
When R is C  =sulfaguanidine

When R is  =sulfadiazine

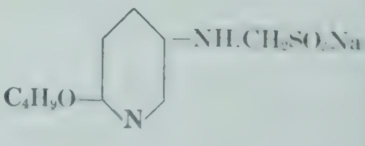
When R is  =sulfamerazine

When R is  =sulfasuccidine (succinylsulfathiazole)

Many similar compounds have been and are being used. Importan among these other compounds is promin [$CH_2OH—(CHOH)_4—CH{\cdot}SO_3Na{\cdot}NH{\cdot}C_6H_4—SO_2$] which has been used with promisin results in the treatment of tuberculosis. A new class of tuberculostatic substances, described by Feinstone also appear to be promising.[25]



Promine



New Compound

Several important principles underlying the action of antimicrobial substances are exemplified in the sulfa drugs.

First, while not inactivated to the same extent by extraneous organic matter as phenol or $HgCl_2$, the sulfa drugs nevertheless lose much of their antimicrobial property in contact with blood, serum, etc.

Second, these drugs are rather specific in their action on various microorganisms. In general they are like cationic dyes in being more effective against gram-positive bacteria than against gram-negative, but, as usual, there are "notable exceptions," *e.g.,* sulfadiazine and sulfathiazole are valuable agents in treating infections due to the gram-negative gonococcus and meningococcus. Sulfaguanidine and sulfasuccidine are not so soluble in the body as the others, tend to remain in the intestine, and to be excreted only in the feces. They are therefore especially useful in treating bacillary dysentery (gram-negative); sulfapyridine is more effective than sulfanilamide against lobar pneumonia, and so on. In using any of them, therefore, the specific efficacy of the drug against the particular organism causing an infection *must* be considered.

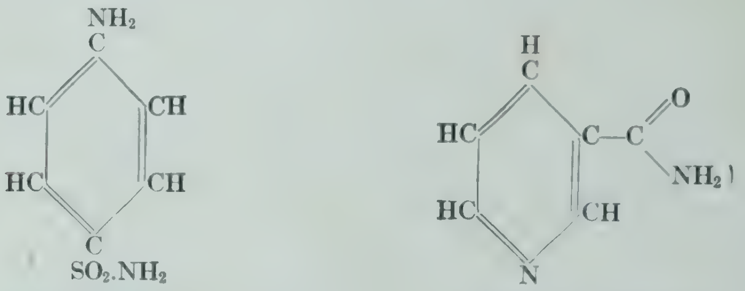
Third, these drugs, like dyes, mercury, penicillin and numerous other antimicrobial agents act almost entirely as bacteriostatic agents. They prevent growth of bacteria, especially streptococci, pneumococci and pathogenic *Neisseria* in the body but do little to kill those already there. As soon as the bacteriostatic agents are withheld the bacteria can continue to grow. Therefore, administration of the drugs must continue till the patient is able to drive out the bacteria himself by means of phagocytosis or antibody formation, or both.[26]

Fourth. Drugfastness. It is a general observation that if various microorganisms are kept in contact with small, sub-lethal concentrations of many substances (various salts, phenol, penicillin, sulfa drugs, etc.), the organisms become adapted and often highly resistant to the effects of the toxic substances. This resistance is called *drug-fastness.* Some even become wholly dependent on the drug. They are, in effect, microbial drug addicts.[40, 41]

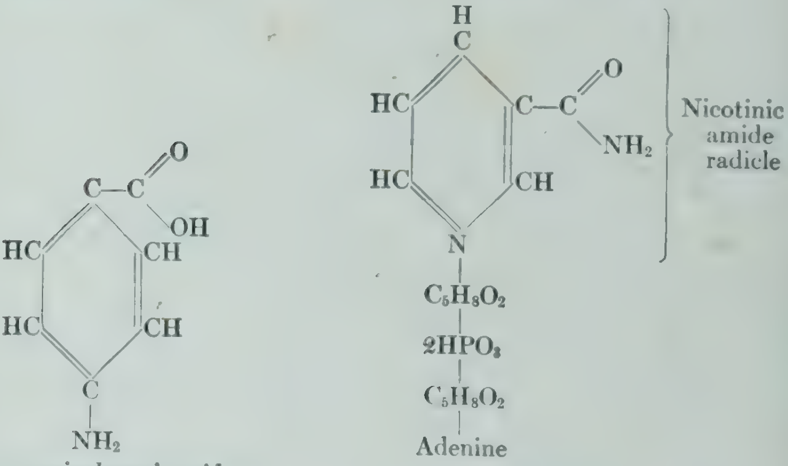
It is a question whether drug-fastness represents survival and growth of very resistant individuals pre-existing in small numbers which dominate the microbial population after the susceptible majority die off; or whether contact with the unfavorable agent induces mutations in the susceptible cells (see chapter on heredity of microorganisms, page 177).[42]

From a practical standpoint, in chemotherapy the drug-fastness or natural resistance of any organism of a supposedly susceptible species should be determined by tests involving cultivation of the patient's own strain of organism in media containing therapeutically feasible concentrations of the drug. If the organism resists the drug, treatment with that drug is contraindicated.

Mode of Action. Studies on the mode of action of sulfonamide drugs and related substances have yielded much information, but no solution which is acceptable to all. The work to 1943 was admirably summarized by Henry[21] and interested readers are referred to that work. A theory widely held[27] is that competitive or antagonistic substances form combination with special enzyme systems.[28, 29, 30] For example, sulfanilamide, etc., and nicotinic acid are chemically similar in some respects (Fig. 113). The two substances seem to have similar chemical affinities and to compete with each other for the same position in the synthesis of certain vital enzyme molecules. Either sulfanilamide or nicotinic acid amide can fill a certain position in the structural make-up of coenzyme I or II (see page 295). Nicotinic amide is a normal part of the coenzyme I and II molecules, while sulfanilamide, which can fit into its position, interferes with the function of the coenzymes and prevents growth. *Para-aminobenzoic* acid and sulfanilamide are also chemically related and nicotinic amide may be derived from para-aminobenzoic acid by molecular rearrangement. Para-aminobenzoic acid may be essential to the construction of coenzyme. Whether this is the exact situation or not, para-aminobenzoic acid has a neutralizing effect upon the bacteriostatic action of the sul-



Sulfanilamide *Nicotinic acid amide*



Para-aminobenzoic acid *Coenzyme I*

Fig. 113. Diagrams showing relationships between bacteriostatic and anti-bacteriostatic compounds and the position of nicotinic acid amide in coenzyme I.

fonamide drug, analogous to the neutralizing effect of —SH compounds on $HgCl_2$. Para-aminobenzoic acid (5 mg. per cent) is therefore sometimes added to culture media when attempting to isolate bacteria such as streptococci or gonococci from blood or pus of patients being treated with sulfonamide drugs.

However, substances unrelated to para-aminobenzoic acid have since been shown to neutralize or antagonize the effects of sulfonamide drugs (methionine, glucose, procaine[31], urethane, etc.). On the other hand, these may exert some other influence, and the action of para-aminobenzoic acid may still be as described above, at least in many organisms. There may be in some cases, if not all, a reversible reaction between the drug and certain enzymes[32], or inhibition of cocarboxylase, etc.[33, 34]

Antagonisms have been found between other essential substances like nicotinic acid and substances closely related to them chemically and able to take their place in the cell, but not physiologically competent. The phenomenon, sometimes called *competitive antagonism*, is apparently illustrative of a general biological principle. Other examples are the neutralization of the antimicrobial action of certain quinones by vitamin K,[35] and the reciprocal action of uracil vs. thiouracil, and thymine vs. thiothymine.[36]

Sulfonamide Inhibitors of Bacterial Origin. Many bacteria produce substances which inhibit the action of sulfonamide drugs. These inhibitors may be demonstrated on nutrient agar plates containing the drug by showing that the heavy, concentrated growth resulting from a "spot inoculation" of a species resistant to the drug permits a localized, satellite-like growth of colonies of a sensitive species of organism dispersed generally over the surface of the plate. Except for colonies appearing as satellites around the spot inoculation of the inhibitor-producing species, the sensitive organism does not grow at all.[37] This phenomenon is well illustrated in Fig. 114. It does not occur on agar without sulfonamide drugs.

It is of interest to note that drug-fast (drug-*adapted,* see page 225) strains of bacteria have not been found to produce more inhibitor substance *after* adaptation than before. Another important point is that in mixed infections, as in wounds, one organism, even though harmless in itself, may interfere with therapy against another because it may produce an inhibitor of sulfonamides.

Sodium Azide. An interesting and useful antimicrobial agent for laboratory purposes is sodium azide (NaN_3). This, unlike most of the substances so far discussed, has an inhibitory effect mainly on gram-negative bacteria rather than on gram-positive species. It has been used for facilitating the isolation of fecal streptococci from water (see page 581), for isolating other streptococci and *Erysipelothrix rhusiopathiae*[38] from infectious processes.

Lichtstein and Soule[39] showed that sodium azide markedly inhibits catalase activity under experimental conditions, a fact which probably explains its action on many species, especially strict aerobes, since catalase is necessary to protect the organisms from their own waste product, H_2O_2. The same authors showed that sodium azide definitely interfered with oxygen uptake of facultative aerobic species.

Detergents. In recent years the problems of disinfection have become closely associated with those of cleansing because many of the

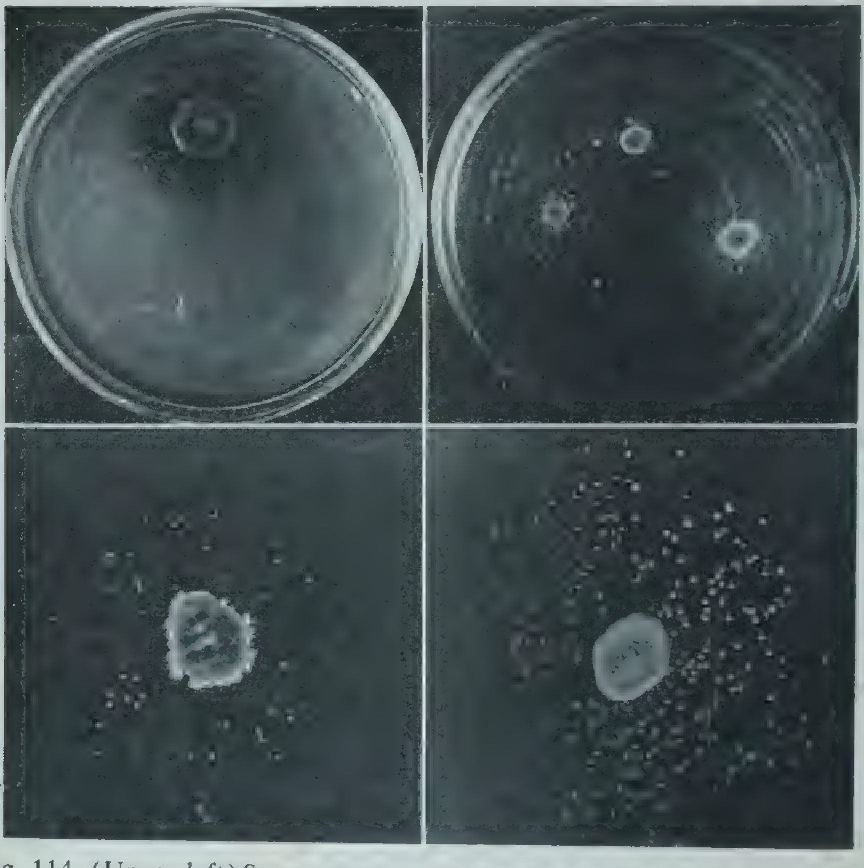
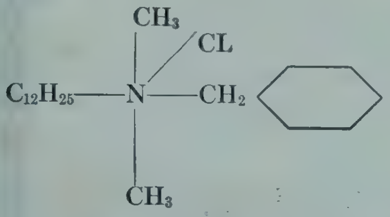


Fig. 114. (Upper left) *Streptococcus pyogenes* as satellite about *Pseudomonas aeruginosa.* (Upper right) *Shigella sonnei* as satellite about *Staphylococus aureus* and *Escherichia coli.* (Lower left) *Streptococcus pyogenes* as satellite about *Staphylococcus aureus.* (Lower right) *Staphylococcus aureus* as satellite about *Salmonella typhimurium.* (Pike and Foster, J. Bact., vol. 47.)

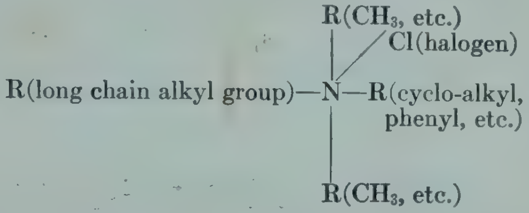
best disinfectants have been found to have also the properties of soaps or detergents.

Soap-like Disinfectants. A new class of disinfectants was discovered in 1936 by Domagk[13] who described the properties of dodecyl-dimethyl-benzyl ammonium chloride. This is a quaternary ammonium salt, and is representative of a large number of similar compounds which have since been developed commercially as disinfectants and detergents.

These compounds have formulas similar to that named above:



or



In general, the more effective are those in which the long alkyl group is a chain of 12 to 16 carbon atoms, while the less effective are of lower molecular weight.[44] The germicidal efficacy of many organic compounds is greatly affected by chemical structure and increases, in any class of compounds such as alcohols, resorcinols, etc., up to a point, with molecular weight, beyond which point it decreases with increased molecular weight.[17, 18]

Since there are many hundreds of detergent disinfectants now available and more are being marketed constantly, it is not feasible to discuss all of them. Each has its own advantages and disadvantages. As a class the quaternary ammonium halides and another group of related compounds having soap-like properties and represented by sodium lauryl sulfate ($C_{12}H_{25}OSO_3Na$), cetyl pyridinium chloride[45] $N—C_{16}H_{33}—Cl$, and cetyl trimethyl ammonium bromide[46, 47] have certain important properties in common. They are not designed for internal use as are drugs like penicillin, the sulfonamides, etc. Their properties in general are:

(1) Actively microbicidal in fairly high dilutions;
(2) not highly irritating or toxic;
(3) not corrosive and do not stain or discolor;
(4) not ill-tasting or malodorous;
(5) readily soluble;
(6) stable per se but are partly inactivated in the presence of organic matter or colloids;
(7) relatively inexpensive;
(8) powerful surface tension reducents; therefore good wetting agents.

Because of their surface properties these compounds act much like soap to emulsify fats and oils and thus they act as good detergents. They promote foaming or lathering and consequently are found in various laundry powders, dish-washing preparations, shampoos, dentifrices, hand washes, etc. Besides being strongly antimicrobial they have the advantage over soap (a weak disinfectant) as detergents in that they do not form insoluble compounds in acidic waters, or with calcium or other minerals in "hard" water, and so do not form deposits or "scum" (insoluble calcium soaps, etc.), or make glass cloudy as does ordinary soap. Soap, however, is not only very useful as a detergent but preferable for many purposes, especially bathing.

Cationic and Anionic Surface-active Detergents. In the examples given it will be noted that there are two types of compounds: those in which the organic radicle is a cation, and those in which the organic group is the anion. These compounds are therefore classified as *cationic* or *anionic surface-active detergents* or disinfectants. The relationship of both types of compound to acidic and basic dyes and other anionic and cationic compounds important to the microbiologist has been mentioned previously (see pages 42 and 118). The cationic compounds appear to be the more generally effective. Some of the surface active detergents are nonionic, *i.e.,* they did not ionize. The mechanisms of their antimicrobial actions are not completely understood but are believed to be analogous to the action of sulfonamides, etc.[48, 49, 50]

As pointed out above, these various surface active compounds appear highly microbicidal by certain *in vitro* tests. However, much caution may be necessary in interpreting the test data, because at least some of them have the property of causing microorganisms to become sticky and to stick to solid objects, and together in clumps, thus producing an apparent reduction in numbers, partly through agglutination and not entirely through actual killing.[51] The method of testing has a very marked effect on the apparent germicidal value.

Relative Strength and Value of Disinfectants. One often hears a disinfectant spoken of as "strong" or "weak" or "mild." These terms are inexact and convey different meanings to different people. To one they mean a "disinfectant odor"; to another, pain on application to a scratch; to still another, corrosive action; to another a pretty color. Rarely does the untrained person think of disinfectants in terms of *bactericidal* strength or toxicity. He has to judge them solely by objective properties. Actually, the value of any substance as a disinfectant depends on a number of factors, important among which are its effect on various materials (discoloration, corrosion, irritation of tissues or toxicity if for use in contact with body tissues), surface tension, cost, and effectiveness or bactericidal action under the conditions surrounding its use. Not all disinfectants are equally effective, and some of them are more effective against some bacteria than against others. Some are effective in the test tube but not in organic matter; some are effective in the vapor phase but not as liquids (see chapter on microbiology of air, page 626).

The effectiveness of many disinfectants may be estimated by mixing them with cultures of certain bacteria and measuring the time required for the substance to kill the organisms. If this is done under carefully standardized conditions, *i.e.*, using a constant quantity of culture medium of a stated composition, a fixed temperature, and a suspension of measured numbers of certain bacteria of known and constant resistance, the results will be quite accurate and reproducible.

The Phenol Coefficient. This is based on a comparison of the effectiveness of the tested disinfectant with that of pure phenol under conditions carefully standardized as noted above. The result obtained is called the *phenol coefficient* of the tested disinfectant. A procedure widely used in this country is that of Ruhle and Brewer,[52] known as the U. S. Food and Drug Administration (FDA) method. In this procedure 5-ml. amounts of a series of dilutions of the disinfectant to be tested (here called X) are placed in a row of tubes of standard size. A similar series of dilutions of pure phenol (here called P) of 1:80, 1:90, and 1:100, is prepared. The temperature of all is brought to 20° C. in a water bath. To each tube is added 0.5 ml. of a standard culture of *Salmonella typhi* having a resistance to phenol such that it is killed in ten minutes by the 1:90 dilution. At intervals of 5, 10 and 15 minutes, a standard loopful (4-mm loop of no. 23 B and S wire) is transferred from each tube, in successive rotations, to tubes containing 10 ml. of sterile broth of standard composition.*

After forty-eight hours of incubation at 37° C. growth in the broth tubes is recorded. The phenol coefficient is the ratio of the highest dilution of X not killing the organisms (evidenced by growth in the corresponding broth tube) in five minutes, but killing in ten minutes (evidenced by no growth in the broth), to the corresponding dilutions of P. The values obtained are tabulated as follows:

		5 Minute Subcultures	10 Minute Subcultures	15 Minute Subcultures
Phenol dilutions	80	—*	—	—
	90	+*	—	—
	100	+	+	+
"Unknown" dilutions	350	—	—	—
	400	+	—	—
	450	+	—	—
	500	+	+	—
	550	+	+	—
	600	+	+	+
	650	+	+	+

* + = growth; — = no growth.

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* Peptone (Armour's special for phenol coefficients)	10 gm.
Beef extract	5 gm.
NaCl	5 gm.
H_2O	1,000 ml.
*p*H	6.8

In this experiment, the 1:90 dilution of P failed to kill in five minutes but killed all the *S. typhi* cells in ten minutes as compared with X which did the same in a dilution of 1:450. The ratio of X to P is $\frac{450}{90}$, or 5, the FDA phenol coefficient of X.

The determination of phenol coefficients requires a high degree of skill. The result itself has very definite limitations. For example, a disinfectant dissolved in distilled water may have a phenol coefficient as high as 50. However, it may be wholly ineffective if applied in the blood, or used in contact with matter such as pus, saliva, feces, milk, etc., as these may combine with the disinfectant and remove it from the bacteria. Further, it may have a coefficient of only 2 or 3 when tested against some other organisms, such as *Micrococcus aureus.* When a substance is said to have a certain phenol coefficient, the limitations of the method must be kept in mind.

Other factors affecting the phenol coefficient are the composition and *p*H of the broth and variations in the organism. Most important, since the comparison is made with pure phenol, the method is applicable only to water soluble substances chemically related to phenol or acting on microorganisms in the same way. However, in practice, many sorts of disinfectants are improperly evaluated in terms of phenol coefficient.

It is important to note that many substances do not *kill* in ten minutes but merely prevent growth of the test organisms in the broth subcultures. The apparent killing and seemingly very high phenol coefficient by some mercurials, for example, can be prevented if transfers are made to thioglycollate broth which inactivates the mercury. Growth may then occur in all tubes and the phenol coefficient be nil.

In order to overcome some of these difficulties the Food and Drug Administration Laboratories make tests of commercial disinfectants with cultures of other organisms and under circumstances designed to imitate actual situations. Thus, tests are often conducted in the presence of blood serum. For testing insoluble or oily products, special methods are used which are intended to approach practical conditions of use. Among these are the filter paper methods (wet and dry) and the agar plate (plain and cup) methods. The following partial descriptions of some of these are taken directly from Circular No. 198, U. S. Department of Agriculture.[52, 53]

"The Wet Filter-Paper Testing Method. The wet filter-paper method is a germicidal test rather than a test of inhibitory properties. It is used when the substance to be tested is not soluble or completely miscible with water, or for substances that are to be used in high concentration, such as soaps, tooth paste, suppositories, dyes, dusting powders, salves, and ointments.

"No. 2 Whatman filter paper is cut into pieces about 0.5 cm. square and sterilized, then impregnated with *Staphylococcus aureus* by immersion in a 24-hour broth culture of the organism. The wet inoculated squares are then placed in the liquid or solid substance to be tested in such a way as to be completely covered and in intimate contact. At the end of 5 minutes, 10 minutes, 15 minutes, 1 hour, or any other desired length of time, the wet papers are removed with a sterilized, stiff, platinum wire bent at a sharp angle to form a hook and placed in 10 cc. of sterile broth. After as much of the disinfectant as possible has been removed (in the case of sticky substances, the needle must be used to aid in freeing the squares of adherent germicide) the squares are retransferred to a fresh tube of sterile broth (10 cc.) and the tubes incubated at 37° C. for 4 hours, when they are observed for evidence of growth.

"**The Agar-Plate Method.** The agar-plate method is a test for inhibitory properties and is used for substances remaining in contact with the body in the absence of serous body fluids, examples of which are salves, dusting powders, creams, plasters, pads, adhesive tape, catgut, and suppositories. The test organism ordinarily used is *Staphylococcus aureus*. The agar is of the same composition as that previously described for carrying stock cultures of the test organism. Strains of staphylococci may vary greatly in susceptibility.[54]

"Fifteen to twenty cc. of agar are melted and cooled to 42°–45° C. To this is added 0.1 cc. of a 24-hour broth culture of the test organism. The inoculated agar is then poured into a sterile Petri plate and allowed to harden. As soon as the agar has hardened the test substance is placed in intimate contact with the surface of the agar. If a salve, it is first warmed just sufficiently to soften it and thus secure a complete peripheral contact. As a control, warmed sterile petrolatum may be placed on another portion of the plate. The plates are incubated 24–48 hours under porcelain tops at 37° C. and then are examined for evidence of inhibition. If the preparation is antiseptic or inhibitory, a zone of clear agar will be noted around the place where the substance has been in contact and the width of the zone will indicate the diffusibility of the inhibitory (antiseptic) agent. If there is no inhibition, growth of the test organism will be observed adjacent to and even under the test substance" (Figs. 115 and 116).

Cup-Plate Method. For liquids small circular depressions are made in the agar by inserting glass rods or small vials about 1 cm. in diameter to a depth of about 2 mm. before the agar solidifies and withdrawing them after it is solid, leaving a little "cup" in the agar to hold the fluid to be tested.

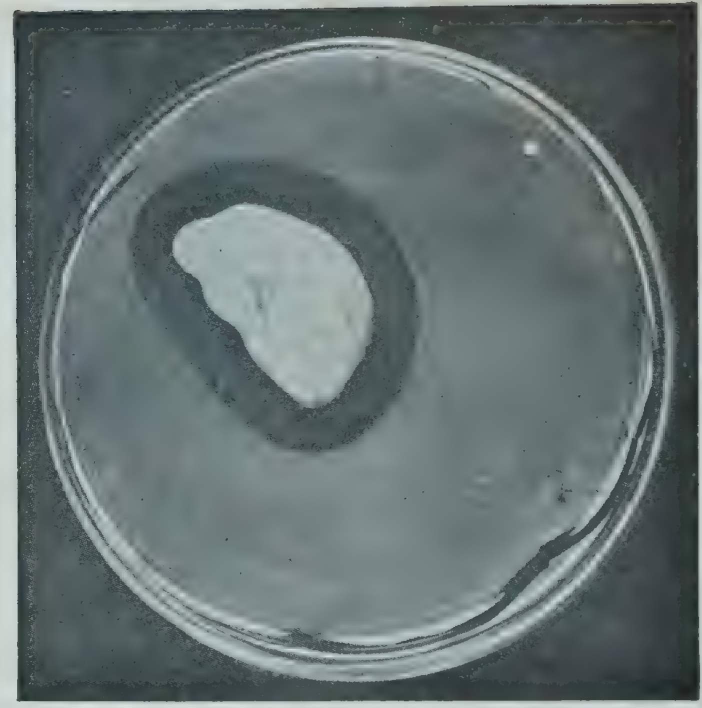

Fig. 115. Agar-plate method showing good antiseptic inhibitory properties and good disffusion, clear dark zone. The rest of the plate is cloudy with myriads of *Staphylococcus* colonies. (U. S. Depart. of Agriculture, Circular No. 198.)

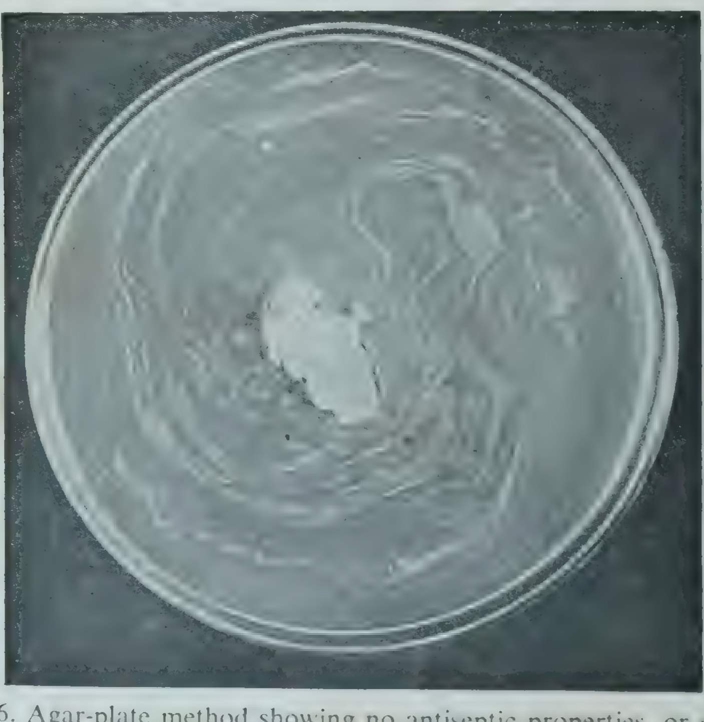

Fig. 116. Agar-plate method showing no antiseptic properties, or evidence of diffusion. The entire plate is clouded with billions of minute *Staphylococcus* colonies which have grown unhampered by the "antiseptic" being tested. (U. S. Depart. of Agriculture, Circular No. 198.)

As pointed out by Tobie and Ayres,[55] there is not necessarily any correlation between cup or agar plate methods and phenol coefficients. The physical conditions requiring diffusion through agar as compared with action in a tube of distilled water obviously measure different properties; so also does the longer time of exposure in the agar plate, as compared with 5 to 15 minutes in the phenol coefficient method.

Toxicity of Germicides. One of the great problems in the selection and use of germicides for use in contact with living tissues is the avoidance of excessive toxic effects. Several methods are available to test the toxicity of germicides. The results are sometimes expressed as *toxicity index, i.e.,* ratio of minimal toxic dose to minimal effective germicidal dose.[56, 57] One may determine the toxic dose of a germicide in various ways, for example, by observing the smallest quantity necessary to stop completely the action of phagocytes in a test tube in a given time.[58, 59] Other methods measure the inhibitory or lethal effect of the tested substance on various tissues,[60] while still others measure the respiration quotient of tissue cells in contact with the germicides.[61] One method[62] is based on observing the survival time of chick embryos into which the tested substances are injected. A time-honored, simple and effective method also, for many substances, is the direct trial on living animals.[63] Good examples of the type of procedure used are in the bibliography.[64]

Probably no single test gives a true result, and species of bacteria, as well as of tissue cells, vary greatly in their susceptibility to different germicides. For any disinfectant, the toxicity index will therefore be different for different tissues and bacteria.

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Chapter 12

STERILIZATION AND DISINFECTION

3. Antibiotics

Probably everyone has had the experience of finding a "lost" object directly in front of him, or even in his hand, after spending some time hunting for it in obscure places. Similarly, for a long time scientists sought after species of harmless microorganisms which would kill or prevent the growth of pathogenic ones so that we might "fight fire with fire," so to speak, and prevent or cure disease through the activity of harmless species.

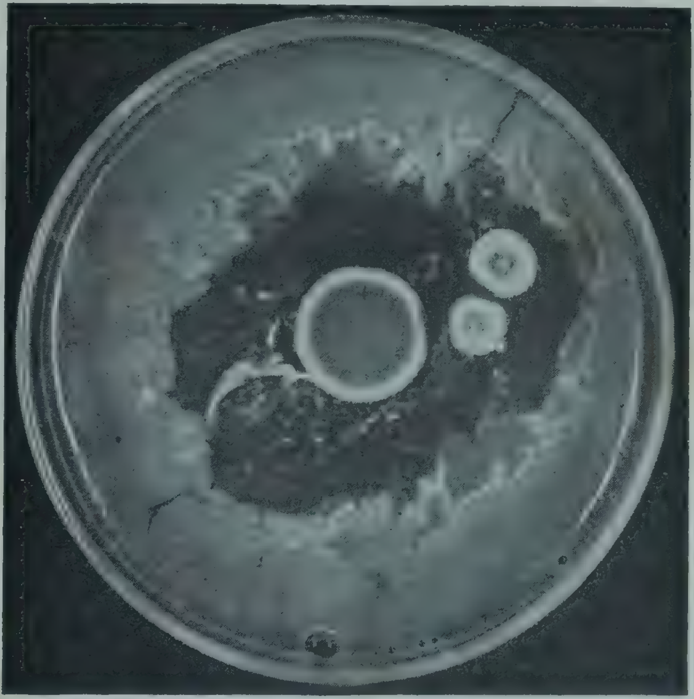

Fig. 117. Colonies of *Penicillium notatum* (center), showing surrounding zone of bacterial inhibition. This is the phenomenon noted by Fleming in 1929 which led to his discovery of penicillin. (Merck & Co., "The Story of Penicillin," 1944.)

The final solution of the problem lay on the laboratory doorstep, as it were, stumbled over and complained of as a contamination by the microbiologist on his way to and fro. For years microbiologists have known that certain environmental microorganisms got into their cultures as contaminants and suppressed the growth of the species which they desired to cultivate. They had seen wide, clear spaces devoid of growth, around certain colonies in culture plates containing bacterial and mold growth. These clear spaces were, of course, due to some antimicrobial substance given off by the colonies surrounded by clear spaces. But the phenomenon was so commonplace and attention so fixed on other problems, that the significance of the observations was overlooked until Fleming, in 1929,[1] appreciated its possibilities and acted upon the basis of his idea.

PENICILLIN

The organism which first attracted Fleming's attention was one of the common molds, *Penicillium notatum* (see Chapter 22), and it occurred to him to experiment with its antimicrobial action. He investigated not only the actively growing mycelium on plates (Fig. 117), but the broth in which the mold had been growing for several days. By passing the broth cultures through filters he removed the mold filaments and so was able to study the activity of the growth products alone as they occurred in the broth. What he found was that the clarified broth contained a highly potent antimicrobial principle, the activity of which was readily demonstrated in contact with sensitive cultures.

Fleming realized the practical possibility of his discovery[1] but was not in a position to develop it more fully and for some years it remained relatively unknown. Attention was called to it in 1935 by Reid[2] who made a rather complete investigation of it, but still it awakened little interest.

Realizing that many pathogenic organisms are destroyed in the soil relatively rapidly, Waksman[3] suggested that the search for an effective antagonistic microorganism be carried to the soil. In 1939 Dubos[4] found organisms (*Bacillus brevis*) in bogs, which produced valuable antibacterial substances (gramicidin, tyrothricin, etc.). But these were too toxic for internal use. His discoveries reawakened interest in Fleming's discovery and work on penicillin was begun in England on a large scale by Florey, Chain, Abraham, and others (the Oxford group) in 1940.[5, 6] Since that time penicillin has played a role in the most titanic war in human history; the most prodigious piece of cooperative research ever organized (prior to atomic research); in a great industrial development; in the most complete control over a variety of diseases ever achieved by man in a short time; and, because of its impact on venereal diseases, in a tremendous new social and moral trend, the possibilities of which are still only partly realized.

Production and Uses. Production of penicillin involves cultivation of mold under conditions most favorable to growth *and* penicillin production. The two are not necessarily inseparable. Growth *and* penicillin production are best at around 24° C. At 37° C. no growth occurs. Penicillin production is best at a *p*H between 7 and 8. As the mold is strictly aerobic, exposure to air is essential.

Commercially, three methods are at present in use:

1. Surface growth on shallow layers of broth in large flat bottles.
2. Growth on the surface of a finely divided material (usually bran) kept moist with nutrient fluid and aerated in large trays.

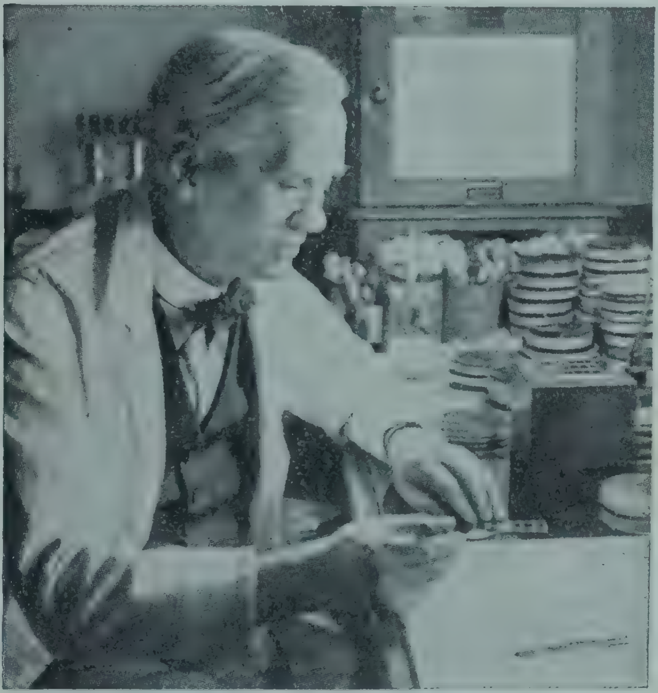

Fig. 118. Sir Alexander Fleming the discoverer of Penicillin (from Les antibiotiques antimicrobiens et la pénicilline by De Montmollin, B. et De Montmollin, A., Mme. A La Baconnière-Neuchâtel).

3. Submerged growth in vigorously aerated tanks holding thousands of gallons of medium.

The large-scale utilization of any of these methods has proven a major engineering problem.

In all methods suitable medium is inoculated with spore suspensions previously prepared by cultivation of *Penicillium notatum* on a medium like Sabouraud's agar (see page 374). During the seven to fourteen days of incubation the mold excretes at least three substances of importance: (*a*) the yellow pigment *chrysogenin,* which must be removed by adsorption with charcoal; (*b*) *penicillin;* (*c*) *notatin* or *Penicillin B* (this occurs especially if the acidity of the medium is too great).

A substance called *pyrogen* is also found in the culture medium and is undesirable since it causes the patient's temperature to rise when injected intravenously. It is removed with the pigment. Pyrogen is probably present in many culture ingredients and may or may not be produced in large amounts by the mold growth.

In the submerged-growth process much heavier and more rapid growth and penicillin production are obtained since the mold grows throughout the tanks and not on the surface alone. Constant agitation and aeration are necessary. This is the most generally used method at present (Fig. 119).

Medium. Different formulae are doubtless in use by various manufacturers but basically they are similar and are mainly modifications of the following, which is one of the best for submerged cultures:[7]

Corn steep liquor*	40	ml.
Lactose monohydrate†	27.5	gm.
$NaNO_3$	3.0	gm.
$MgSO_4 . H_2O$	0.25	gm.
KH_2PO_4	0.50	gm.
$ZnSO_4 . H_2O$	0.044	gm.
$MnSO_4 . H_2O$	0.020	gm.
Glucose monohydrate‡	3.0	gm.
Water ad	1000	ml.

Synthetic media have been proposed, but have not been widely adopted for commercial use, since growth is slower, use requires special care in aeration, and in construction of machinery.[10] They have a value in laboratory studies on metabolism, etc.

After about seven days fermentation is complete and *p*H rises to 8.0 or above and penicillin production ceases.[11]

When no more penicillin is being formed, the masses of mold growth are separated from the culture fluid by centrifugalization and filtration and the process of extracting the penicillin from the clear fluid begins.

Full details regarding extraction and purification cannot be included here.

In one process of purification the clarified broth is acidified (*p*H 2 to 3), and shaken briefly with amyl acetate. The amyl acetate is separated and shaken with buffer solution (*p*H 7.0). Some of the pigment is removed by adsorption on animal charcoal and filtration. The solution is then shaken with ether and the ether extract passed through an adsorption column containing $Al(OH)_3$ which adsorbs penicillin in its upper and middle portion. The pyrogen and pigment are taken out in other parts of the column. This yields its penicillin to ether again, whence it is finally extracted with buffer solution (*p*H 7.2). A liter of culture may yield a few milligrams of pure crystals. Further details on production may be found in literature cited.[12–16]

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* Corn-steep liquor is a by-product of the distilling industry, being the water used to soak (steep) the corn prior to fermentation. It contains various growth factors (vitamins), proteins, carbohydrates, etc., and is one of the best sources of nutriment for penicillin production by many strains of the mold.

† For some strains, cotton seed meal is said to be superior to corn steep.[8] Corn steep is acid and must be neutralized.

‡ Brown sugar is also recommended for some processes[9], and is said to be superior to lactose and glucose. It contains useful organic impurities.

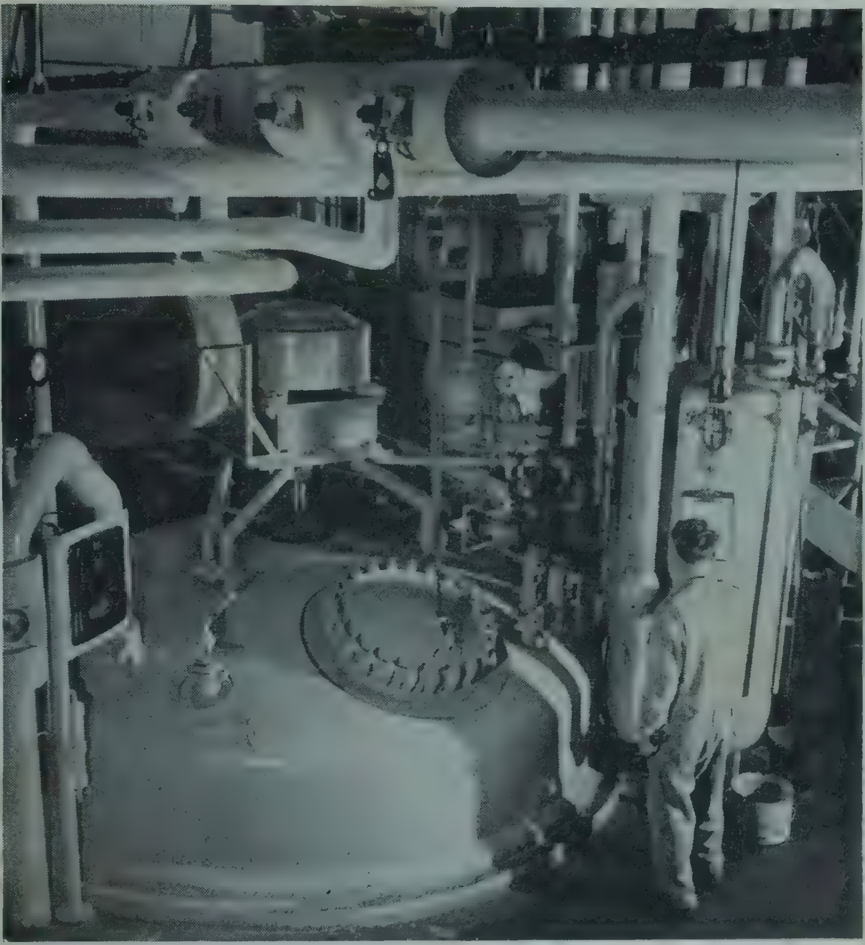


Fig. 119. Like a steel igloo the top of a 9,000 gallon fermentation tank rises above the floor level of the penicillin plant at Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y. Small tank at the right contains chemicals to prevent excessive foaming of the liquid containing the mold. (Peter Winkler, Lederle Laboratories, Inc.)

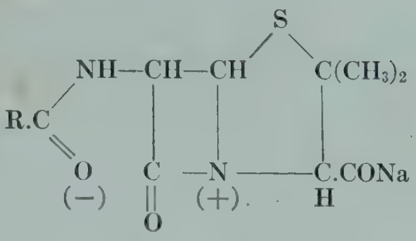
Properties. The "pure" substance (as will be shown later, in reality a mixture of chemically related substances called penicillins X, G, F and K) occurs as an acid and is generally used as the sodium salt, although calcium penicillin has some special uses. Penicillin is readily soluble in water and alcohol but the latter inactivates it. As a dry powder it is sold in vials and in this form it is only slowly inactivated by light, but is injured by heat and storage in warm places. It will retain its potency fairly well in the refrigerator at 10° C. or lower. Once in solution it deteriorates rapidly at room temperature but will remain potent for several days if kept at icebox temperature, 4° C. to 10° C.[17] It is quickly destroyed by acid.

Among the most valuable properties of penicillin are its non-toxicity for man and animals even in relatively large doses, whereas its potency is such that a concentration of 0.000001 gm. per ml. of some preparations will exert marked bacteriostatic effects. It may be administered subcutaneously, intravenously or locally. Unfortunately the acidity of gastric juice destroys it unless large amounts of bicarbonate or other alkali are given.[18] Penicillin has the advantage also that it does not act, as do phenol, iodine, bichloride, etc., by non-specific combinations with *all* proteins so as to produce coagulation or degradation.

Mode of Action. Penicillin probably acts as a specific cell poison (for certain species only) possibly by the inactivation of one or more components of their respiratory enzyme system in a manner analogous to sulfonamide drugs.

The action of penicillin may be very closely associated with the dismutation of pyruvic acid. The inhibition, by penicillin, of pyruvic acid dismutation, is reversible by washing the penicillin from the cells. This reversible bacteriostatic action is strongly suggestive of the reversible action of bichloride of mercury and dyes. It is of especial significance also that penicillin inhibits the purified enzyme.[19]

Chemistry and Varieties of Penicillin. In view of expense and difficulties involved in commercial production of natural penicillin, the desirability of ascertaining as soon as possible its chemical structure with a view to artificial synthesis on a commercial scale is obvious. As soon as studies along these lines were begun, the existence of four different penicillins F, G, X, and K was recognized because of differences in structure of crystalline penicillins from different sources, and the fact that American samples yielded phenylacetic acid on hydrolysis (G), while British samples yielded 2-hexenoic acid (F).[20, 21] Clinical studies[22, 23] carried on at the same time indicated their presence in varying proportions in different lots of "natural" penicillin. It was found that the various penicillins are strong, monobasic acids, *p*H about 2.8. The general chemical structure provisionally assigned to them is:



The R varies in the different penicillins as follows:[24]

Nomenclature		Side chain
British	U. S.	(R)
I	F	— penteryl $CH_3.CH_2.CH{=}CH.CH_2$—
II	G	benzyl $C_6H_5CH_2$—
III	X	*p*-hydroxybenzyl $OH{-}C_6H_4.CH_2$—
IV	K	*n*-heptyl $CH_3(CH_2)_6$—

Laboratory synthesis of penicillin was announced shortly after the formula was known.[25] The substance actually produced was the triethyl-ammonium salt of benzyl penicillin (G). A number of other substances closely related, which also had antibiotic properties, were produced experimentally during the research, thus revealing the possibility that *better artificial penicillins and other antibiotics* may be made than are available from natural sources.

Some Properties of Penicillins F, G, K and X. In the very early preparations of natural penicillin apparently much F, G and X were present, with relatively little K. In clinical studies later it was found that natural penicillin, being then commercially produced, apparently lost much of its effectiveness.[22, 23] This was afterwards shown to be due to variations in the natural product such that much K was occurring in the mixture. Now, K is very active *in vitro,* but in the body it is very rapidly inactivated or excreted by liver and kidney[26] and by a thermostable serum component.[27] Thus its value for clinical purposes is low. All of the penicillins are excreted at rather high rates, but none so fast as K.

At present the most generally useful and readily available penicillin is G; X is least rapidly excreted, more expensive, and best for streptococcal and pneumococcal infections.[28] G is used in treating syphilis.

It is interesting to note that natural impure penicillin greatly increases the activity of any of the pure F, G, X or K penicillins when mixed with them. It may contain some substance still unknown, which conditions the activity or stability of the pure crystals.[29]

Various Strains of Penicillium Notatum. The early studies on penicillin were made using Fleming's strain of *P. notatum* which for a time was thought to be unique. It was later found that some "wild" strains of this organism were better producers of penicillin, and that other, closely related organisms, especially *P. chrysogenum,* also produce penicillin. These findings led to a very extensive search for better strains of *P. notatum* and for penicillin-producing strains of other organisms. Many thousands of cultures were tested and it was found that the penicillin-producing property is widely distributed in the *P. notatum-chrysogenum* group and that some strains are better than others, especially for the various methods of production, *i.e.,* some were good in tanks, not so good on surfaces, and vice versa.

Later, naturally-occurring variants of the best strains were tested and it was found that strains might be of two types: a conidial or C type, producing relatively less mycelium, many conidia and much penicillin; and an M or mycelial type, producing much mycelial growth, but relatively few conidia and little penicillin. Much care is, therefore, exercised to maintain cultures in the C condition and to save stocks of spores of these strains for "seed." A few strains of natural variants were markedly superior to the parent strains. The search for a "super" strain continues.

As will be shown in the chapter on microbial variation and heredity (see page 176) irradiation with ultraviolet or x-rays of sex cells may induce mutations or variant strains having markedly different metabolic properties from the original. This has been found to be so with *Penicillium notatum,* and irradiation has furnished many thousands of "artificial" variants for testing. The method has yielded some very potent penicillin-producing variants.

It is especially interesting to note the usefulness of this method in inducing the development of variants which produce large amounts of one or another of the various penicillin isomers, F, G, X or K.[30] Penicillin X has great therapeutic value, and artificially induced variant strains producing much X and relatively little F, G, and K, especially under tank conditions, were sought and found.[31] Penicillin G is particularly valuable for some clinical purposes, especially syphilis, and is more readily available than X, and doubtless strains, natural or artificial, producing predominantly G can be found or developed. Some of the penicillin now sold commercially is pure, crystalline penicillin G.

Factors Inhibiting Penicillin. *Penicillinase.* Many microorganisms produce an enzyme, *penicillinase,* which rapidly destroys penicillin. In the presence of such species the action of penicillin is suppressed or eliminated.[32]

However, penicillinase is very valuable. Just as it is important to be certain of the sterility of sulfonamide drugs before using them for local application, so it is necessary to determine the sterility of penicillin for similar use or for intravenous injection. Contaminating organisms in penicillin could well escape detection by ordinary cultural methods because the penicillin itself might inhibit their growth until they got into the body tissues. By using purified penicillinase to inhibit penicillin, tests for sterility of the drug are made possible. The enzyme is also useful for identification of penicillin since it is specific in its action.

There appears to be no relation between the penicillin-resistance of an organism and its power to produce penicillinase. Cultural conditions such as *p*H and temperature greatly affect production and activity of penicillinase so that organisms producing it may appear sensitive to penicillin under some conditions and not under others. Among the species producing penicillinase are *E. coli,* members of the genus *Bacillus* and some species of *Shigella.*[33, 34, 35]

Clarase and Other Enzymes. Several amylolytic (starch-digesting) enzymes have been found apparently to destroy penicillin and have been used for some of the purposes mentioned above. Among these are taka-diastase and clarase, both produced by the mold, *Aspergillus oryzae.* Contaminating bacteria (*Bacillus mesentericus*) may be active in these enzymes.[36]

Cysteine. Difficulties in production of enzyme preparations for inactivation of penicillin have been largely overcome by the discovery that the amino acid cysteine (hydrochloride) accomplishes the same thing, is inexpensive and can be autoclaved.[37, 38, 39]

Serum factors may also inactivate penicillin, especially penicillin K.[27, 40]

Acquired Resistance to Penicillin (Drug Fastness). It has been found that many species of organisms, gonococci, staphylococci, meningococci, etc., originally susceptible to penicillin and other antibiotics become resistant if held in contact with low and increasing concentrations of the drug. The same phenomenon has been described in connection with sulfonamide drugs. The development of resistance does not appear to depend on development of the power to produce penicillinase.[41–44] As previously noted, in therapy, laboratory tests of the resistance of the patient's organism to the drug being used should always be made.[45]

Uses of Penicillin. The value of penicillin as a selective bacteriostatic agent in laboratory bacteriology was recognized by Fleming and others, for the isolation of resistant organisms such as the whooping cough and influenza bacilli from throat cultures,[46] and for suppressing bacterial growth in studies of viruses and rickettsiae and pleuropneumonia organisms (see Chapters 46, 49, 50).

The clinical uses of penicillin are beyond the scope of this discussion, but some of the common infectious organisms against which it is more or less effective are listed here.

Highly susceptible

B. anthracis
Various clostridia including *Cl. tetani* and *Cl. welchii.*
D. pneumoniae
Neisseria sps., including gonococci and meningococci.
Micrococci
Streptococcus pyogenes, etc.
C. diphtheriae (in vitro)

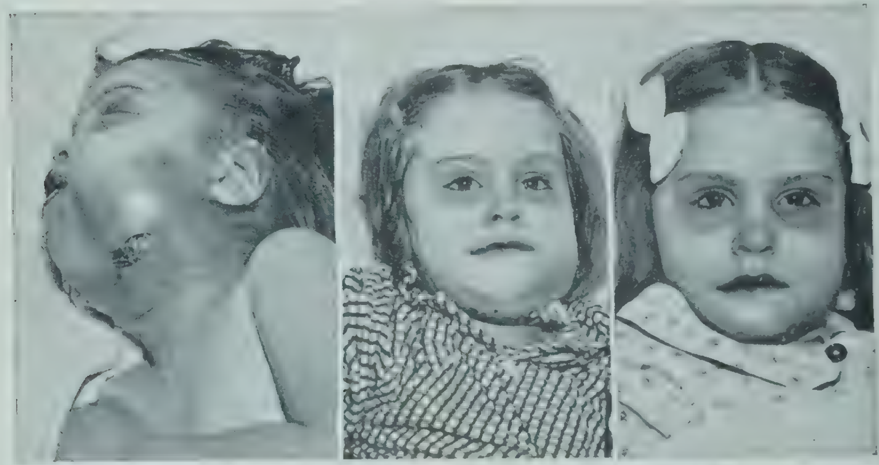


Fig. 120. Child with facial and orbital cellulitis, shown at beginning of treatment, ninety-six hours after beginning treatment with penicillin and nine days after treatment was instituted. (Herrell, Proc. Staff Meet., Mayo Clin., vol. 18.)

Moderately susceptible

Borrelia of relapsing fever
Various alpha ("viridans") type hemolytic streptococci.
Leptospira of Weil's disease.
Treponema pallidum.

It is generally ineffective in dysentery, typhoid, and salmonella infections, influenza and pertussis infections, tularemia, brucellosis, and virus and rickettsial diseases.[63]

Assay and Standardization. *The Oxford or Florey Unit of Penicillin.* In order to express potency accurately a standard of reference was early established by Florey and it is spoken of as the Florey or Oxford unit. The original Oxford unit is the least amount of penicillin necessary to inhibit completely the growth of a certain strain of *Staphylococcus aureus* in 50 ml. of a standardized meat extract broth. A certain lot of penicillin of carefully measured potency was put aside to be used as a basis for comparison in the assay of new lots of penicillin. Later, a method of measuring potency of penicillin was based on the principle of the agar cup method of testing disinfectants as described in another section (see page 233). It is called the cylinder-plate method (see following paragraphs). The Oxford unit was then expressed in terms of the width of the zone of inhibition of growth in the agar around the cylinder. One milliliter of the original standard, containing one unit as previously defined, was found, by the cylinder-plate method, to produce a zone of inhibition exactly 24 mm. in diameter.

In 1944 an international conference was held to establish standards and test procedures. At this meeting (London, October 16–19) a certain specimen of pure crystalline sodium penicillin G was adopted as a point of reference (international standard) for all standardization. An international unit was then defined as 0.6 micrograms of the *international standard* sample, this quantity having been found to equal 1 Oxford unit. A calcium salt, carefully standardized, was then distributed as an international "working standard." Of the international working standard 1 unit is contained in 2.7 micrograms. Certain strains of *Staphylococcus aureus* for assay work were designated as standard.

Methods of Assay. There are various procedures for assaying the potency of penicillin. All are really modifications or adaptations of only three basically different methods. These are: the cylinder-plate method;[47, 48, 49] the dilution method; and the agar streak plate method.[50]

Sources of error in all of these methods are:[51] varying proportions of the various penicillins in any sample and differences in susceptibility to each, of the test organism; presence of traces of carbohydrate in medium or sample—this will activate *notatin;* traces of bactericidal impurities in the sample such as organic solvents; variations in composition of broth or density of agar; variation of the test organism.[52]

In the *serial dilution method* serial dilutions of the sample are made in standard broth in culture tubes, which are then inoculated with a uniform amount of the test organism. Turbidity or its absence are noted and may be measured by means of a photoelectric turbidimeter.[53, 54] The turbidities are compared with a dilution series made with a reference working standard. The method is laborious and requires sterilization of the sample which destroys some of its potency.

Agar streak methods are rapid, do not require a sterile sample, permit the use of several test organisms at once, and can be used to test substances not dissolved in water. They are most useful as preliminary assays of crude material. Dilutions of the substance to be tested are placed in a series of Petri dishes. Into these are poured

10 ml. of melted and cooled agar which is thoroughly mixed by tilting the plates back and forth. After the agar has hardened, the plates are marked into several sectors, on each of which is streaked a different test organism. After incubation, the dilution of the antibiotic inhibitory to each organism is recorded (see Fig. 126).

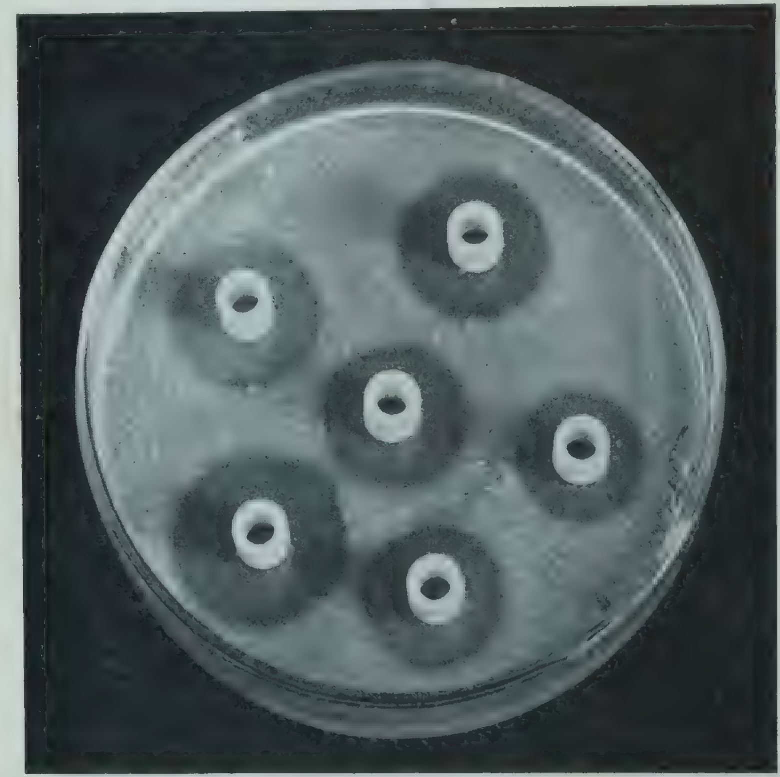


Fig. 121. Cylinder plate standardization of penicillin. The culture plate contains a medium, uniformly and heavily seeded with staphylococci or spores of *B. subtilis*. In each cylinder was placed a measured quantity of penicillin-containing extract. Following incubation under standard conditions, growth of bacterial colonies gave the medium the pebbled, gray appearance seen everywhere except around the cylinders. Here, growth has been inhibited by penicillin. Measurement of these zones of inhibition permits standardization of penicillin in Oxford units by comparison with zones produced under identical conditions of test by standard solutions of known unit strength. (Therapeutic Notes, March, 1944, Parke, Davis & Company.)

Cylinder-plate Method. Probably the most generally satisfactory method of assay is the cylinder-plate method. A small sterile cylinder with a perfectly flat edge, made of porcelain or glass is sealed by touching it, while slightly heated, to the upper surface of the already-inoculated solid agar and leaving it there. The solution to be tested is placed in the cylinder (Fig. 121). Diffusing into the agar, the penicillin solution reveals its potency by the width of the zone of inhibition. This may be read accurately by the use of calibrated viewers (see Fig. 122).

Suspensions of *B. subtilis* spores are the most satisfactory test inoculum,[48] because the edges of the zones of inhibition are very sharp and clear. Factors affecting the width of the zones are depth, density and *p*H of the agar, and amount of inoculum. An improvement over the Petri dish and a device for placing the cylinders on the agar surface have been introduced (see Figs. 123, 124).[55, 56]

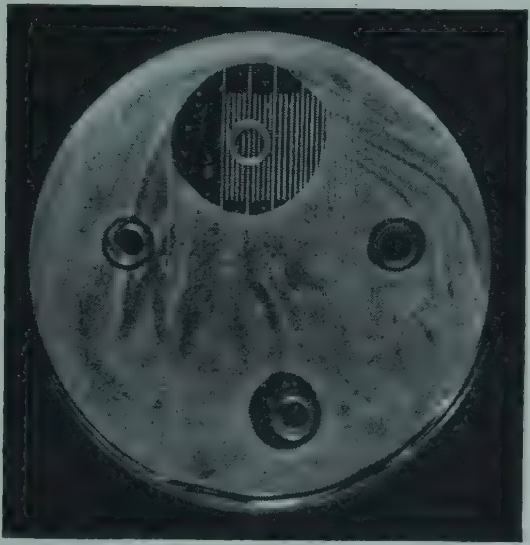


Fig. 122. The diameter of the inhibited zone—30 mm. wide in this case—is readily observed with the aid of the ruled plate and reflected light of the penicillin reader. (Fisher Scientific Co.)

A convenient modification of the cylinder-plate method is the use of discs of thick filter paper in place of the cylinders. The sterilized discs are saturated with the sample and placed on the agar. The method has the advantage of ease of manipulation both of discs and plate.[57, 58] The method gives more acceptable results in assaying penicillin content of blood.[59]

Many modifications of the three basic techniques described above, as well as completely new procedures, have been published, review of which is beyond the scope of this book. New methods are being constantly published.[51] The problem of assaying mixtures of antibiotics has been discussed by Rudert, Kenner and Foter,[60] Kavanagh,[61] Schmidt, Ward and Coghill,[52] and Higuchi and Peterson,[62] the latter using a microbiological method.

STREPTOMYCIN

The discovery of streptomycin dates only from 1944 and followed naturally in a long series of researches by Waksman and his collaborators into the numbers and kinds of antagonistic microorganisms in the soil.[64] The source organism produces conidia and aerial mycelia and is one of the order Actinomycetales, genus *Streptomyces* (*S. griseus*)—whence streptomycin (Fig. 125).

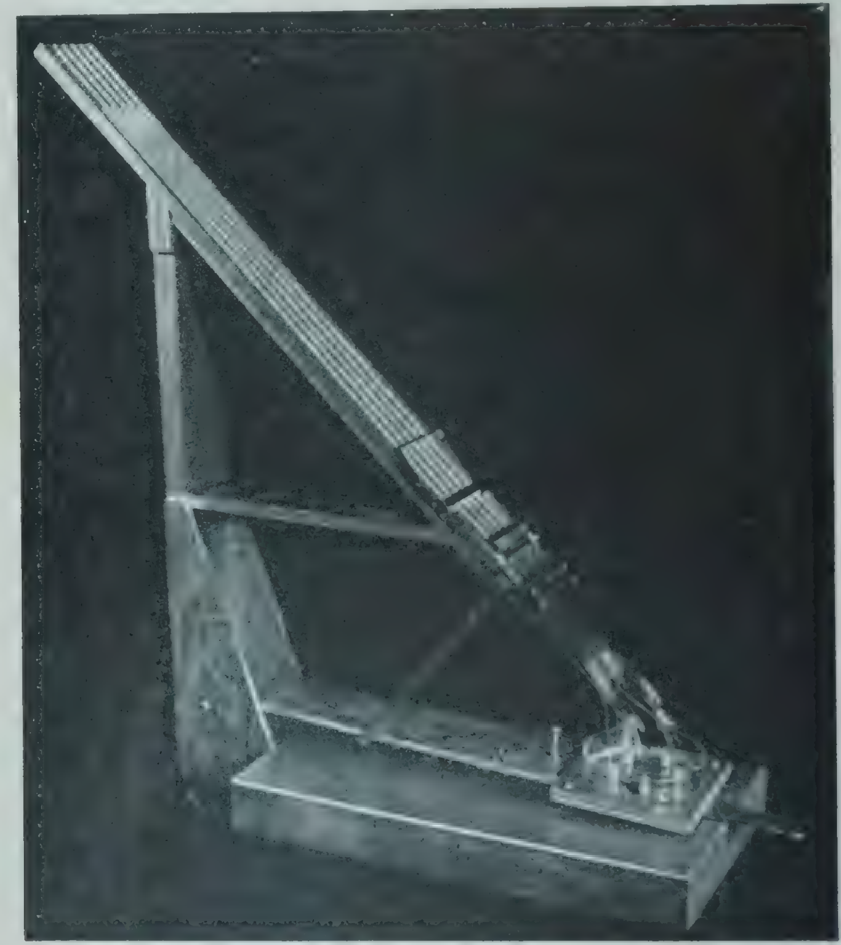


Fig. 123. Cylinder-placing device. (Reeves, M. D. and Schmidt, W. H., in J. Bact., vol. 49.)

The general principles underlying the production, purification, assay and standardization of streptomycin are closely analogous to those pertaining to penicillin, differing only in respect to cultural details, and technical procedures appropriate to the organism and substance involved. One of the best media consists of:

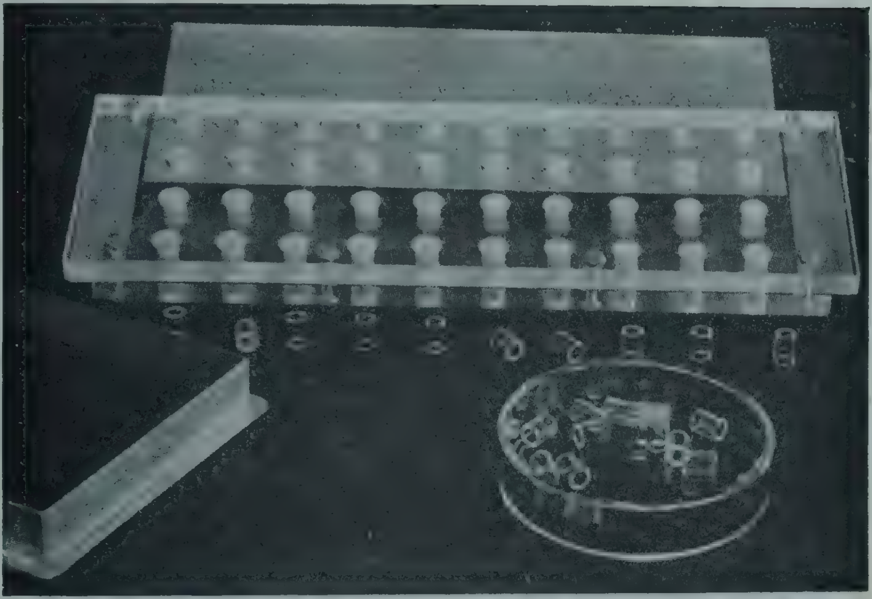


Fig. 124. View showing lucite guide, cylinders, and metal rim. (Beadle, G. W., Mitchell, H. K., and Bonner, D., in J. Bact., vol. 49.)

Fig. 125. Dr. Selman A. Waksman and his assistant, Mr. Albert Schatz, selecting strains of the streptomycin-producing culture of *Streptomyces griseus*.

Ingredient	
glucose	10 gm.
peptone	5 gm.
meat extract	5 gm.
NaCl	5 gm.
Water	1,000 ml.
*p*H	6.5–7.0

Aeration of the culture is essential for rapid streptomycin production, which is at its maximum in two to three days. The medium becomes gradually more alkaline. Various strains of *Streptomyces griseus* are more or less active producers of the drug.

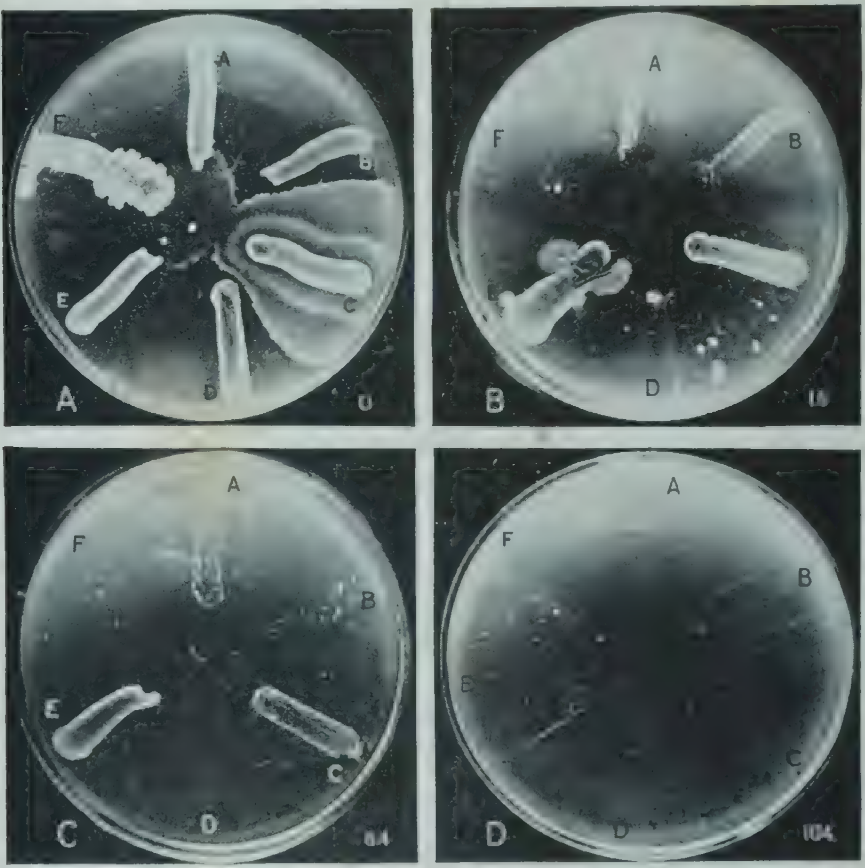


Fig. 126. Antibacterial activity of streptomycin. A. *Escherichia coli*. B. *Salmonella typhi*. C. *B. proteus*. D. *Klebsiella pneumoniae*. E. *Bacillus pyocyaneus*. F. *Mycobacterium tuberculosis*. The letters refer to streaks of growth on the plates. The numerals indicate micrograms of Streptomycin per ml. of agar. (Courtesy of Merck & Co., Inc.)

After separation of mycelium from the fluid, the fluid is treated with activated charcoal (*Norit*) which adsorbs the streptomycin. The charcoal is separated from the fluid, washed with alcohol to remove impurities and then with dilute acidified alcohol. The latter removes the streptomycin from the *Norit*. The acid-alcohol solution is then neutralized and filtered to remove further impurities. Ether separates the alcohol from the water and the streptomycin is left in the water in fairly concentrated form, from which it may be removed by precipitation with acetone or vacuum desiccation. Crystalline streptomycin is obtained by further processing.

The unit of streptomycin is analogous to the unit of penicillin but not exactly the same. The S unit, originally proposed, is the least amount which, when present in 1 ml. of nutrient medium, just inhibits a standard strain of *Escherichia coli*. Because of the inconvenient smallness of this quantity, an L unit was devised, being similar to the S unit but referred to a volume of 1 liter instead of 1 ml. Later, a G unit was defined on the basis of activity of crystallin material and was found to be about 1 million S units. A new unit is based on crystallin material, of which 1 gamma is equal to 1 unit (1 Oxford unit of penicillin=0.6 gamma).

For assay of streptomycin, the streak-plate method serves as a preliminary determination (Fig. 126), and more accurate definitions of potency depend on the cylinder-plate method, using a certain strain of *S. aureus,* or the paper disc method.[65] The sterility of streptomycin may be determined by inactivating the drug with cysteine as in testing penicillin, or by semicarbazide.[68] A broth dilution method has also been described.[66, 67]

The formula of basic streptomycin is tentatively given as $(C_{10}H_{19}O_{7-8}N)_n$. The drug is much more stable than penicillin, both in dry form and in solution. In the refrigerator the solutions retain potency for months, and at 37° C. for about two weeks. Boiling for ten minutes produces about 50 per cent loss of potency.

The activity of streptomycin in vitro is bactericidal (rapid killing) in high concentrations; bacteriostatic (inhibits growth) in less concentrations. Its scope of action is in some respects broader than that of penicillin, being active against a variety of gram-negative and gram-positive organisms, some of which are listed below.[69]

Gram-positive
Mycobacterium tuberculosis
Staphylococcus aureus
Diplococcus pneumoniae
Streptococcus, sp
Bacillus subtilis (and related species)
Erysipelothrix
Not:
Clostridia

Gram-negative
Salmonella typhi
Pasteurella tularensis
Klebsiella pneumoniae
Brucella abortus
Proteus vulgaris
Salmonella schottmülleri
Hemophilus pertussis
Hemophilus influenzae
Pseudomonas aeruginosa

Streptomycin has been used effectively in many infections with gram-negative rods not susceptible to penicillin or sulfonamides. It is of relatively low toxicity in therapeutic doses and is not nearly so rapidly destroyed in or excreted from the body as penicillin. Some toxic effects (vertigo, deafness, etc.), have been described. Unlike penicillin, it is not destroyed in the gastrointestinal tract and may be orally administered, although it is not much absorbed from the gastrointestinal tract. Streptomycin, in order to be therapeutically effective, must be present longer and in greater concentrations than penicillin. Details on clinical usage are given in the literature.[70]

Factors affecting the activity of streptomycin are:

(1) Salt concentration; NaCl, KCl, Na_2SO_4, Na tartrate, phosphates, and NH_4 acetate interfere with its effect on certain organisms in proportion to their concentration.
(2) Age of culture and phase status.
(3) Number of organisms.
(4) Composition of medium.

The mode of action of streptomycin is not entirely clear. It appears to be definitely related to aerobic respiration and to oxygen uptake.[70, 71, 72, 73, 74]

No enzyme such as penicillinase has been reported, but resistance (drug fastness) of organisms may be induced.[75, 76, 77]

OTHER ANTIMICROBIAL SUBSTANCES

During the exploitation of Fleming's discovery of penicillin an enormous volume of work was done in efforts to discover other antimicrobial substances of biological origin. The search was not unsuccessful. Many such substances have been found, the sources ranging from bacteria, molds and actinomycetes, to higher plants and animals.

ANTIBIOTICS FROM MOLDS

Source	Inhibiting Substance	Susceptible Organisms
Penicillium cyclopium	Penicillic Acid (1913)	Gram-positive and gram-negative bacteria
Penicillium notatum	Penicillin (1929)	Gram-positive bacteria, gonococci, meningococci, spirochetes, actinomycetes, clostridia, Corynebacterium diphtheriae
Penicillium chrysogenum	Penicillin	
Aspergillus flavus	Flavicidin Flavicin (may be identified with Penicillin)	
Aspergillus giganteus	Gigantic Acid (may be identified with Penicillin)	

Source	Inhibiting Substance	Susceptible Organisms
Aspergillus parasiticus	Parasiticin (may be identified with Penicillin)	
Penicillium citrinum	Citrinin (1931)	Gram-positive bacteria chiefly
Gliocladium fimbriatum Aspergillus fumigatus	Gliotoxin (1936)	Kills gram-positive bacteria, Salmonella paratyphi, Shigella dysenteriae, fungi
Aspergillus fumigatus	Fumigatin (1938)	Chiefly gram-positive bacteria
Aspergillus flavus	Aspergillic Acid (1940)	Chiefly gram-positive bacteria
Aspergillus clavatus	Clavacin (1942)	Inhibits and kills gram-positive bacteria, some fungi
Aspergillus claviformis	Claviformin	(Identical with Clavacin)
Aspergillus fumigatus	Fumigacin	(Identical with Clavacin)
Penicillium patulum	Patulin	(Identical with Clavacin)
Penicillium notatum	Penatin (1942)	Kills gram-positive and gram-negative bacteria
Penicillium aurantio virens	Puberulic Acid (1942)	Gram-positive bacteria
Aspergillus fumigatus—Mut. Helvola	Helvolic Acid (1943)	Chiefly gram-positive bacteria
Chaetomium cochliodes	Chaetomin (1943)	Gram-positive bacteria
Aspergillus fumigatus		Mycobacterium tuberculosis
	ANTIBIOTICS FROM BACTERIA	
Pseudomonas aeruginosa	Pyocyanase	Dissolves gram-positive and gram-negative bacteria
Pseudomonas aeruginosa	Pyocyanin	Kills gram-positive and gram-negative bacteria; fungi
Bacillus brevis	Tyrothricin	Gram-positive bacteria
Bacillus brevis	Gramicidin	Inhibits gram-positive bacteria only
Bacillus brevis	Tyrocidin	Kills gram-positive and gram-negative bacteria; fungi
Bacillus subtilis	Subtilin (1943)	Gram-positive bacteria only
Bacillus brevis	Gramicidin S (1944)	Inhibits gram-positive and gram-negative bacteria
	ANTIBIOTICS FROM ACTINOMYCETES	
Actinomyces antibioticus	Actinomycin A (Waksman, 1941)	Chiefly gram-positive bacteria, fungi
Proactinomyces gardneri	Proactinomycin (Gardner, 1942)	Gram-positive bacteria, gram-negative cocci
Actinomyces lavendulae	Streptothricin (Waksman, 1942)	Gram-negative and gram-positive bacteria, fungi
Actinomyces griseus	Streptomycin (Waksman, 1944)	Gram-negative and gram-positive bacteria, Mycobacterium tuberculosis, actinomycetes

Fig. 127. Antibiotic Substances. (Eli Lilly and Co.)

No attempt will be made here to review this very extensive field. Most of the substances so far found are of little practical value at present, being mainly too toxic for therapeutic use. Others are too readily inactivated in vivo; limited in potency or scope of activity; not readily soluble, or have other undesirable properties. Some are identical with each other. Some of them are useful in research and special work of various sorts. Many made newspaper headlines at the time of their discovery. A list of several antibiotics, with data on sources, activities, etc., is given above. Literature on several of the substances listed will be found in the bibliography.[78–98]

A much publicized antibiotic is *chloromycetin,* produced by a certain species of *Streptomyces.* Chloromycetin is active against a wide variety of bacteria and, unique among antibiotics, has given promising results in the treatment of rickettsial infections, and even in certain of the virus diseases.[87, 88, 89]

Several recently studied antibiotics have been derived from bacteria closely related to, or identical with, *Bacillus subtilis.* Among the more promising of these are *subtilin* and *bacitracin.* The latter is active against a number of gram-positive bacteria and has been studied as a possible drug in dealing with syphilis. Another bacterial product is *polymyxin* derived from *Bacillus polymyxa.* Polymyxin differs from many other antibiotics in its specificity for gram-negative bacteria. Most others, except streptomycin, are effective chiefly against gram-positive organisms.[90, 91]

Two other bacterial products recently accorded some publicity are *bacillomycin,* closely related to subtilin and produced by *B. subtilis;* and *aerosporin* derived from an aerobic spore-forming rod much like, if not identical with, *B. subtilis.* Bacillomycin is distinguished by its activity against pathogenic fungi such as those causing "Athletes' Foot" and related conditions. Aerosporin resembles polymyxin in being active against gram-negative organisms such as the typhoid and whooping cough bacilli. It is also said to be bactericidal in its action, whereas most antibiotics are bacteriostatic.[92, 93, 94, 95, 96, 97]

An interesting summary of the status of knowledge of many antibiotics is to be found in the review by Florey.[98]

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Chapter 13

ENZYMES AND MICROBIOLOGY

When biochemists and cell physiologists began to investigate microorganisms it was revealed that these lowly creatures have a physiological chemistry as complex as the cells of more highly evolved beings. It was especially interesting to note that the physiological chemistry of animals, human beings and plants of all sorts, including bacteria and other fungi, is fundamentally alike. Different physiological mechanisms are involved, and a variety of chemical devices are made use of in various species and classes and phyla, but basically all are seen to be essentially the same. The chemical mechanisms involved in growth and energy production of all higher plants and animals are obviously mere elaborations and modifications of processes already "invented" by the lowly autotrophic bacteria.

Many of the physical and chemical mechanisms underlying plant and animal metabolism are fairly well understood and can be observed and controlled experimentally. Among the most extensively investigated mechanisms are those involved in respiration or the utilization of foodstuffs as energy sources. The reactions resulting in synthesis of protoplasm, *i.e.*, the utilization of foodstuffs as building material for the living organism, are constantly being subjected to intensive studies but are not yet so well understood as the energy-yielding or *exothermic* reactions. Both the exothermic and the energy-storing (cell-building or *endothermic*) processes are accomplished largely through the agency of organic catalysts* called *enzymes*. A brief discussion of catalysis and of enzymes at this point may, therefore, be useful in later discussion of microbial metabolism.[1, 2, 3, 4]

Catalytic Agents. If two chemically reactive substances, as for example oxygen and ethyl alcohol, are adsorbed at ordinary temperatures upon the surface of a third inert substance, such as platinum, which has been treated so as to be in a finely divided or colloidal condition, we have a series of reactions as follows:

$$C_2H_5OH + O_2 \longrightarrow CH_3COOH + H_2O$$
$$CH_3COOH + 2O_2 \longrightarrow 2CO_2 + 2H_2O$$

facilitated and controlled by surface concentration or adsorption.

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* In order to understand catalytic agents, the student should review the sections on colloids; surfaces of colloids; surface tension; adsorption.

These reactions take place at an astonishing rate of speed at ordinary temperatures (under 100° C.) in the presence of the platinum, whereas ordinarily oxygen and alcohol combine exceedingly slowly, even imperceptibly, except at ignition temperatures (burning). The platinum does not enter into the reaction, but remains to adsorb more oxygen and more alcohol, and so continues the process of oxidizing the alcohol first to acetic acid and then to water and carbon dioxide. In such a reaction the platinum acts as a *catalytic* agent, and since the whole effect depends on the surface of the platinum, its presence in a colloidal state is essential. In a simple system of this kind we can predict, from a knowledge of the substances and surfaces involved, what the result of a given combination will be. Inorganic catalysts like platinum are simple and constant and are extensively used in industry. In living systems the situation is more complex. Here several organic catalysts called *enzymes* act simultaneously.

Enzymes. An enzyme is an organic catalytic agent, synthesized by a living cell.

All enzymes appear to be protein complexes. Being proteins, enzymes are colloidal in nature, chemically complex, and unstable. They are sensitive to all of the various influences which affect proteins in general. For example, the activity of all enzymes is adversely affected by temperatures above or below their optimum (and usually within the range from about 20 to 78° C.), excessive *p*OH or *p*H, high osmotic pressures, excessive concentrations of substrate* or end products, often by pressures, ultraviolet light, and so on. The action of many enzymes is prevented by specific enzyme poisons, among which are narcotics, cyanides, dyes, H_2S, CO, disinfectants, etc. These poisons are often adsorbed on, or combine with, the catalytically active surface of the enzyme to the exclusion of the usual substrate. Some of these "blocking" actions are reversible, others permanently inactivate the enzyme.

Comparing these statements with what has been said of the effect of various environmental factors on microorganisms, it is clear that, in general, whatever affects enzymes also affects microorganisms (as well as all other living cells), since the physiological chemistry of bacterial and other cells is almost entirely due to, and dependent upon, the action of enzymes or enzyme-like bodies.[1, 2, 3, 4]

The action of enzymes, like that of inorganic catalysts, is to *speed up* chemical transformations, whether exothermic, endothermic or otherwise. In many cases they appear to initiate reactions which would not occur spontaneously to any appreciable degree. Unlike most inorganic catalysts, they appear to form loose but very specific chemical combinations with the reacting substances during the transformations they catalyze. The action of many enzymes is demonstrably reversible, and for this reason the symbols used in equations involving enzyme action generally indicate reversibility. However, in a considerable number of reactions no reverse process is apparent, but this may be due to the conditions under which the reaction occurs. It is important to note that enzyme reactions cease when a certain concentration of the end-products of the reaction have accumulated; *i.e.,* they reach an equilibrium point. If, for example, we place in a solution of amyl butyrate (an ester) a little esterase (an enzyme from the pancreas which hydrolyzes esters), the amyl butyrate is decomposed to butyric acid and amyl alcohol. The decomposition ceases when a certain concentration of the acid and alcohol has been reached. Conversely, if we place the acid and the alcohol together in a beaker with lipase, amyl butyrate is formed, ceasing at a certain concentration of the butyrate. In many instances reactions of this nature *appear* to proceed in only one direction because the equilibrium point is very far in that direction. In other cases one phase of the reaction seems to occur with greater ease than the reverse. Such reactions, although reversible and although they embrace both hydrolytic and synthetic phases, under the conditions of action inside the cell are usually pushed decidedly toward the one or the other phase of the reaction. Otherwise matters would tend to become static and soon reach equilibrium and this is not compatible with life. Thus, in the hydrolysis of amyl butyrate, equilibrium is soon reached in a beaker of water, but in a living cell the products of hydrolysis may be removed by excretion as fast as they are formed and equilibrium may never be reached during life.

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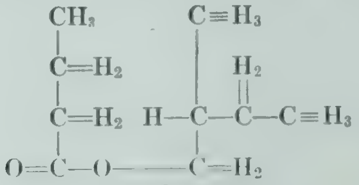
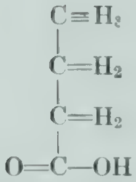
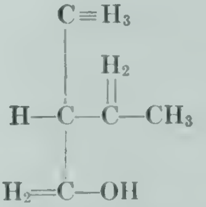
* The material being acted upon by an enzyme.


Amyl butyrate


Butyric acid


Amyl alcohol

Composition of Enzymes. Although many enzymes are easily demonstrated by their activity free from the living cells which produced them, relatively little is known as to their exact chemical composition. One difficulty in obtaining exact information about many bacterial enzymes arises from our inability to obtain large quantities of them in a pure, native state, and to be certain that what is supposed to be a single enzyme is not a mixture of two or more. However, many enzymes of animals, yeasts and other fungi have been obtained in pure, even crystalline, form. The first so obtained was urease; later crystalline pepsin, trypsin, amylase and about forty others were prepared. All that have been purified have been shown to be proteins and to form colloidal solutions. The activity of the enzyme is dependent upon the integrity of the protein molecule and the colloidal state. Hence the sensitivity of enzymes (and of living cells which depend for life on their activity) to disinfectants, heat, etc. (Fig. 128).

Classification and Nomenclature of Enzymes. Early students of physiology, discovering new enzymes, often gave them descriptive names without reference to any systematic scheme of nomenclature. Such names as *pepsin* (the protein-digesting enzyme from the stomach), *trypsin* (the proteolytic enzyme from the pancreas), *ptyalin* (the starch-digesting enzyme in saliva) are time-honored examples of this early method of naming enzymes. Later it became necessary to systematize nomenclature. It is now customary to name an enzyme by adding the suffix "ase" to the substrate or substance acted upon. For example, protein-digesting enzymes are *proteinases, amylase* digests starch (amylum), and so on. Some enzymes are named for their activity. For example, *oxidases* bring about oxidations; *dehydrogenases* cause hydrogen to leave the substrate; *hydrolases* cause hydrolysis.

Specificity. A very distinctive property of enzymes is their *specificity.* This is of almost the same order as immunological specificity (see Chapter 16) and probably depends on similar stereochemical relationships. For example, an enzyme which catalyzes the oxidation of natural *l*-amino acids will not oxidize the *d*-amino acids. Enzymes which hydrolyze proteins will not hydrolyze carbohydrates and vice versa. An enzyme which destroys the carboxyl group of pyruvic and related keto acids will not attack the carboxyl group of acids like acetic acid. Some enzymes act upon *groups* of substances, as trypsin, a proteinase which acts upon proteins in general. Some proteinases are very specific; for example, bacterial *caseinase* acts only on casein and not on other proteins.

The specificity of enzymes supports the view that their role in catalysis is the formation of an adsorption compound. The enzyme temporarily combines with the *substrate* (the substance being acted upon) to form an unstable compound which immediately undergoes the oxidative, hydrolytic, or other change characteristically induced by that enzyme, releasing the enzyme to repeat the process. Thus a very small amount of an enzyme can bring about a large amount of chemical change in a relatively short time. For example, 2 ml. of concentrated extract from pigs' pancreas, containing the enzyme *trypsin* which hydrolyses protein, can decompose approximately 5 pounds of ground beef within five hours or less at 37° C. and a pH of around 7.5. The ratio of substrate (beef) to actual, pure enzyme is well over a million to one.

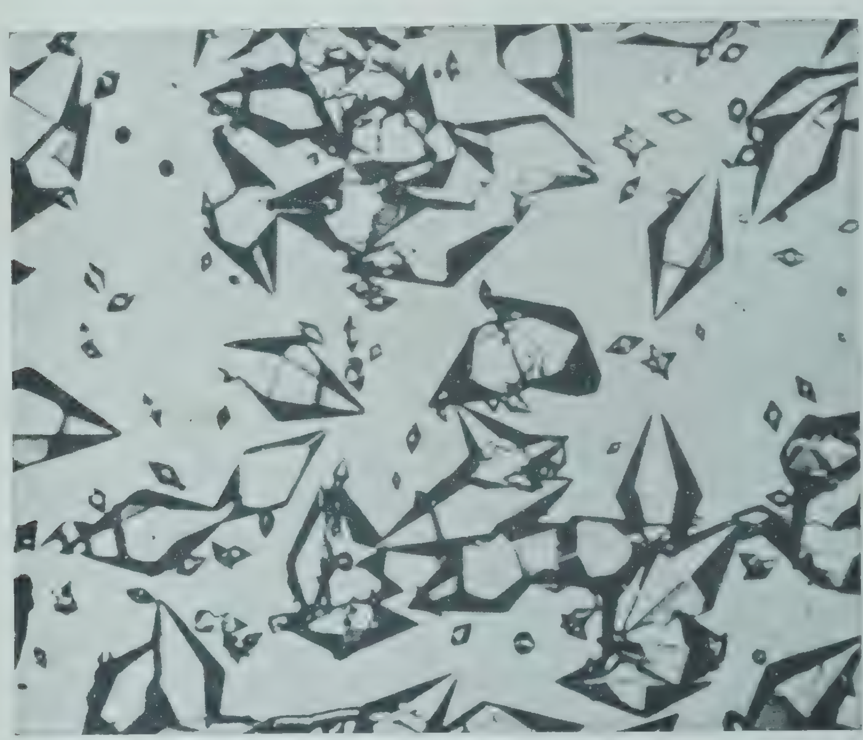


Fig. 128. Crystalline pepsin. (Northrop.)

Distribution of Enzymes. There appears to exist, in one cell or another, plant or animal, an enzyme capable of attacking in some way practically any naturally occurring organic substance and many artificially produced organic substances. It is difficult to find a natural organic compound incapable of supporting some form of life under suitable conditions of moisture, temperature, accessory food sources, etc. However, while all living cells contain or produce enzymes which catalyze their vital chemical processes, all cells do not produce all enzymes. Among microorganisms there are very sharp and clear-cut differences in this respect, and it is partly by means of their specific and characteristic enzymes that we identify and classify bacteria. For example, many intestinal bacteria produce *lactase*, but the typhoid bacillus and related species which cause intestinal disease do *not* produce lactase, a fact which greatly aids in their isolation and identification.

Endo- and Exo-enzymes. Enzymes may exist only inside the cell, or they may be secreted to the exterior environment where they carry on mainly hydrolytic or digestive functions. Enzymes within the cell are intracellular or *endo-enzymes,* while those functioning outside the cell are extracellular or *exo-enzymes.* Most of the exo-enzymes are hydrolases. Enzymes which are not hydrolytic, mainly endo-enzymes, are often grouped as *desmolases.* This group includes *oxidases, dehydrogenases, mutases* (cause a Cannizzaro reaction), etc. Examples of various kinds of enzymes are seen in the accompanying table (Table IV) which gives the nomenclature, action, and other information regarding some enzymes.

Activators. Coenzymes. Some enzymes are first produced in an inactive form called *zymogens* or *pre-enzymes.* These are *activated* by contact with some other agent outside the cell called a *kinase* or activator. Trypsin, for example, exists in the pancreas as trypsinogen which becomes active when it gets into the intestine in contact with a substance called *enterokinase.* Neither substance alone can perform the function of the enzyme. The esterase *phosphatase* is activated by magnesium ions. Pepsinogen is activated by metallic ions, bile salts and a variety of other substances to give it full activity.[6]

Many enzymes seem to be composed of two parts which are inactive separately; (*a*) a nondialyzable colloidal protein and (*b*) a specific, active or prosthetic group or *coenzyme* which is nonprotein and dialyzable. Some coenzymes are readily separable from the protein molecule constituting the colloidal part of the enzyme, others are very firmly attached and it is doubtful whether they may be considered as separate chemical bodies.

The activity and specificity of the entire complex (called a *holo-enzyme*) seem usually to be conferred in great part by the coenzyme or prosthetic group, although this is not always so. In some enzymes specificity appears to depend on the protein portion, while in still others the entire complex is a fairly stable compound and specific activity appears to depend on the entire substance.

Coenzymes play a very important role in all cell metabolism and nutrition. We shall describe some of them more in detail in discussing specific enzyme functions in metabolism.

Cell Surface as an Enzyme. While the enzymes, both extracellular and intracellular, as well as the constituent protoplasm of bacteria are colloidal and therefore possess possibilities for extensive surface reactions, doubt has been raised about the multiplicity of enzymes supposed to exist in bacterial cells. It is possible that many important reactions are brought about by, or on, the external surfaces of the cells themselves. Bacterial cells are so small that they have many of the surface properties of colloidal particles. For this reason it seems not unlikely that the cell itself may accomplish many of the changes ascribed to enzymes, by adsorbing and transforming substances on its own surface, the nature of the transformations depending on the chemical and physical make-up of the cell exterior, its

TABLE IV

ENZYMES (PARTIAL LIST)

Name	Substrate and Action
Proteinases	*Proteins hydrolyzed in varying degrees*
Pepsin	Proteins hydrolyzed to proteoses, peptones, etc.
Trypsin	Proteins hydrolyzed to polypeptides and amino acids.
Gelatinase	Gelatin hydrolyzed.
Fibrinolysin	Fibrin decomposed.
Carbohydrases	*Carbohydrates hydrolyzed*
Amylase	Starches hydrolyzed to maltose.
Lactase	Lactose hydrolyzed to glucose and galactose.
Sucrase	Sucrose hydrolyzed to glucose and fructose.
Esterases	*Esters, fats, etc., hydrolyzed to alcohols and acids*
Lipase	Fats hydrolyzed to glycerol and fatty acids.
Phosphatase	Organic phosphates broken down to phosphate and radicle.
Dehydrogenases	*Hydrogen is activated to leave the substrate*
Succinic acid dehyd.	Succinic acid oxidized to fumaric acid.
Lactic acid dehyd.	Lactic acid oxidized to pyruvic acid.
Glucose dehyd.	Glucose oxidized to gluconic acid.
Mutases	*Cannizzaro reactions; molecular rearrangements*
Aldehyde mutase	2 molecules of aldehyde simultaneously oxidized and reduced.
Phosphoglyceric mutase	Rearrange phosphoglyceric acid to phosphopyruvic acid.
Oxidases	*Oxygen activated to combine with substrate*
Indophenol oxidase	Oxidize cytochrome and indophenol.
Peroxidase	Release active oxygen from H_2O_2.
Polyphenol oxidase	Oxidize di- and tri-phenols, and related compounds.
Carboxylases	*Decompose carboxyl groups*
Pyruvic carboxylase	Carboxyl group of pyruvic and related acids split to CO_2 and R·CH.
Amino acid carboxylase	Carboxyl group of amino acids split to CO_2 and R-NH.
Deaminases	*Split off amino group of amino acids*
l-amino acid oxidase	*l*-amino acids deaminized to NH_3 and aldehyde or keto acids.
Guanase	Guanine oxidatively deaminized to NH_3 and xanthine.

electrical potentials and other factors. This mechanism is not to be thought of as a substitute for the concept of enzymes, but as an additional mechanism for producing metabolic changes. The question is an unsettled one.

Products of Microbial Metabolism. Not all bacteria possess all enzymes. The metabolic compounds resulting from the action of enzymes are, therefore, often characteristic of various species. Indeed, as shown in a previous chapter, it is largely by the study of the products of metabolism that the enzymes are recognized and microorganisms identified. The identification of microorganisms by their metabolic products or peculiarities is extremely important and is more fully discussed elsewhere (see page 189). Some of the products of metabolism, like ethyl alcohol and acetic acid, are very useful and valuable in industry. Others, like diphtheria toxin, are extremely poisonous. Some of them, like nitrates, are indispensable for plant food and are formed in large quantities only by the action of bacteria which inhabit the soil. Indeed, it is to the microorganisms of the soil that the human race owes its existence. If the soil microorganisms ceased functioning, plants would not grow, empires and republics, old order and new order, would fall to dust and proud *Homo sapiens* would pass out of existence.

The Rothen Phenomenon. Observations by Rothen in 1946 and 1947[6, 7] on the action of enzymes and on the attraction of antigens for antibodies* *through an impermeable membrane* up to 1,000 Angstrom units† (A°) thick may necessitate a considerable revision of previously held ideas concerning enzyme action and antibody action. These have always assumed *close physical contact* between antigen and antibody or enzyme and substrate. The essential facts of Rothen's discoveries are shown by three observations:

(1) If an antigenic substance, such as serum albumen, is deposited in a very thin film on a clean, flat metal slide, and is then immersed in a solution containing antibodies specific for that antigen, a layer of antibodies of a definite thickness combines with the layer of albumen. If, on a clean slide, 7 or 8 or more successive layers of albumen antigen are deposited, then the amount of antibody deposited at the surface of the *topmost layer* of albumen, when the slide is immersed in antibody solution, is proportionately *much thicker* than would be expected if the most superficial layer of albumen in actual

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* An antibody is a substance produced in the serum of an animal in response to the entrance of an antigen or foreign protein (e.g., flesh of another species or a bacterium) into the tissues or blood of that animal. Antibodies tend to destroy the foreign protein or antigen. The relationship between antibodies and antigens is specific and closely analogous to the relationship between enzymes and their substrates. (See also Chapter 16.)

† An Angstrom unit is a 1 ten-millionth of a millimeter.

contact with the antigen were the only effective one, which would be according to the older ideas. Obviously the *deeper* layers of albumen exert a definite attractive influence on antibody *at the surface,* even though they may be several hundred A° *below that surface.*

(2) A remarkable feature of this attraction effect of the buried layers of antigen is that their influence can extend not only through the 6 or 7 overlying layers of albumen but, if the whole mass of albumen is covered with an *impermeable film* of plastic ("Formvar"), the effect is not diminished. A coating of metallic film, however, destroys the attraction force. This suggests that the force is electromagnetic in nature.

(3) Several layers of albumen or other antigen are laid on the metal slide. The whole is covered with "Formvar" which may range up to 1,000 A° in thickness. An enzyme capable of hydrolyzing the antigen is then applied at one spot over an area of perhaps 1 sq. cm. After a time the enzyme and the plastic film are removed by appropriate solvents. The slide is now immersed in a solution of antibody. The usual antigen-antibody reaction occurs at the surface, but not at the area previously covered by hydrolytic enzyme. Obviously the enzyme, *completely separated* from the substrate by upwards of about 1,000 A°, by an *impermeable* plastic film, has exerted a *hydrolytic action* (*or influence!*) *across a relatively very great distance.* These facts seem to be well established.

The implications of these findings, if they are finally confirmed, are very far reaching and of the most fundamental sort. The ideas of enzymes as adsorptive, contact catalysts must be drastically revised. All the thermodynamic concepts and the accompanying mathematical computations must be revised. The role of the cell membrane as an enzymatically active surface will assume greater importance, since the necessity for diffusion of substrates through the cell wall in order to be acted on by enzymes will be reduced or removed. Antigen-antibody concepts will need to be changed. Indeed, the full effect of the impact of this revolutionary discovery on present concepts of enzyme action and many dependent and related biological phenomena, including immunity and disease production, and especially on our ideas of the mechanisms of specificity in enzyme and immunological phenomena can hardly be estimated, but it will be enormous (provided it proves to be truth and not an illusion)![8]

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Chapter 14

THE METABOLISM OF MICROORGANISMS

1. Nutrition; Assimilation

The life chemistry of microorganisms is not different in principle from that of other living things. They require water and certain elements common to all protoplasm. Most important are C, H, O, N, P, S, Na, K, Ca, Fe. Some of these elements are obtained from complex organic molecules by means of enzymes which split (hydrolyze, etc.), organic compounds into simpler compounds and elements; some are obtained from simple inorganic substances like H_2O, CO_2, H_2S, $MgSO_4$, and so on.

Whatever the sources from which these elements are obtained, they may be used in two ways. First, they may be *assimilated* in the synthesis of cell substance and reserve food like starch, fat, etc. Such uses of food involve *endothermic reactions* since energy is "built in" to the synthesized substance and stored there. Second, all organisms *dissimilate* (the reverse of assimilate) and bring about oxidations (*exothermic reactions*) of certain of their food materials. These reactions yield the energy used in assimilation or manifested as motion, reproduction, heat and so on.

An analogy is seen between the relationship of assimilation and dissimilation and the building of a column of bricks. Each brick piled up represents a chemical synthetic reaction resulting in the building up of some energy-bearing cell substance such as starch or fat, for example. This is assimilation. Potential energy is accumulated in the fat or column of bricks. When the column of bricks is knocked down, or when the fat is hydrolyzed and oxidized, energy is liberated in the form of heat, motion, perhaps light, etc. The decomposition of the fat or the falling down of the column of bricks represents dissimilation. Further energy is obtained by the living organism through the mechanism of respiration or biological oxidation of the constituents. This is discussed further on.

While it is true that all organisms require much the same elements in roughly similar proportions, the *forms* in which those elements must be provided, *i.e.*, the food or nutritive requirements, vary greatly. Animals all require vegetable or animal tissues, or complex organic derivatives thereof, as foods. Green plants require only CO_2, water and simple inorganic substances, minerals, etc., from the soil. But bacteria and related microorganisms have a great many different kinds of nutritive requirements entirely unlike those of the animal kingdom and not found elsewhere in the vegetable kingdom.

Types of Bacterial Nutrition. Bacteria differ among themselves especially in respect to sources of (*a*) carbon, (*b*) nitrogen, and (*c*) energy. These requirements range from the extremely simple and plant-like (inorganic) to the very complex and animal-like (organic), depending on the species of bacterium. We may regard the autotrophic or prototrophic forms of microorganisms as one extreme, living entirely on inorganic matter and *especially* on inorganic carbon (CO_2). Parasitic heterotrophs with complex and often unknown organic requirements, such as body or tissue extracts and fluids and unidentified vitamins, are the other extreme. Between these are all gradations, with combinations of autotrophic and heterotrophic requirements.

The tendency in nutritive requirements away from autotrophism toward parasitism may be regarded as an evolutionary development involving the loss of synthetic powers. Autotrophs obviously have highly developed synthetic powers, synthesizing fats, carbohydrates, proteins, vitamins, enzymes, etc., which enable them to live independently of other forms of life and without the necessity for parasitism or even saprophytism. Heterotrophs, saprophytes and parasites, depend on other living things to do a certain amount of their synthetic work for them, and to prepare fairly complex molecules such as amino acids, glucose, vitamins, etc., ready formed for them; their needs in this respect vary with different species. ✓

We may list various stages in the scale between the extremes (autotroph and highly adapted parasite) on the basis given above, *i.e.*, carbon, nitrogen and energy requirements, somewhat as follows:

Groups	*Examples*
1. Require inorganic N, C, and non-solar energy source.	Soil autotrophs: *Thiobacillus, Nitrobacter,* etc.
A. As above, but use solar energy.	Photosynthetic autotrophs: Rhodobacteriineae.*
2. May use, prefer, or require inorganic N but require organic C.	Various saprophytes and some parasites: *E. coli, Rhizobium, Azotobacter.*
3. Require organic N and C sources, which may also serve as energy sources.	Various saprophytes and parasites like micrococci.
4. Require organic N and C and other complex compounds including vitamins and unknown tissue components.	Various highly adapted parasites like syphilis spirochetes and diphtheria bacilli.

Such a classification is complicated by the fact that (1) some organ-

* Some of these require organic N or C or both.

isms in several of these groups, especially 1, 2, and 3 have all or nearly all of the properties of their group, but also 1 or more properties of other groups. Usually this overlapping of properties can be seen as the retention of primitive properties; *i.e.,* evolution has changed them in the direction of parasitism in all but a few respects. For example, *Salmonella typhi* has the power to grow in blood and invade tissues, but still retains its primitive ability to live in a wholly inorganic solution with a little glucose (organic C) and tryptophane (organic N). (2) *Many,* probably *all,* groups require a certain amount of *inorganic* carbon as CO_2, some species more than others.[1, 2]

Many organisms at present classed in groups 3 and 4 might be placed in a lower group if we had more exact knowledge of their requirements. For example, we are often forced to supply whole blood or serum or tissues, because we do not know just what specific component of the blood, etc., is actually needed. Many of our complex organic media will probably be replaced eventually with mixtures of relatively simple substances of known composition. This is the aim of much research now being conducted. Such mixtures of known and reproducible composition involving chemically pure substances are called *synthetic media.* A good example is seen in a study by Niven, Washburn and White[2a] on the nutritive requirements of a species of streptococcus.

A series of studies by Starr et al. on nutritive requirements of bacteria pathogenic for plants illustrates the general method used in such investigations. It also illustrates the very simple requirements of some organisms capable of parasitism and pathogenicity, and the lack of synthetic powers of others.[3]

It is desirable to know the exact nutritive requirements of microorganisms for reasons of economy in time and money in their cultivation, but more especially for exactness and reproducibility of media in studies of their growth, chemistry, metabolism, effects of various physical and chemical agents, immunological problems, disease processes, agriculture, industry, variation, heredity, and indeed, all aspects of microbiology. If we do not know the exact composition of the solutions of foods involved, we certainly cannot draw any exact conclusions from experiments in which nutrition and growth are factors.

Inorganic Nutrient Requirements. In addition to the ten elements listed at the beginning of this chapter as known to be essential to the synthesis of protoplasm it seems that several others are also needed. However, many of the latter are effective in exceedingly minute ("trace") amounts. Our knowledge in this respect is incomplete because the trace elements occur, often unknown to us, as impurities in even the purest "C.P." chemicals, in distilled water, etc. They include nearly all the common metals and many of the nonmetals. Some may be essential, some may not be. Because of technical difficulties we cannot readily eliminate them so as to study the effect of their entire absence. The ten mentioned above must be present in considerable amounts and about these we are able to be more definite.

However, it has been possible to study the effects of absence of some metallic cations by removing them from nutrient solutions by means of a cationic exchange column. Their absence often has marked effects on the metabolism of the test organisms, especially on the method of dissimilation of glucose.[4] Apparently, various enzyme systems are activated by the metals, especially manganese and chromium.

Autotrophs. The known inorganic requirements for the synthesis of protoplasm by the autotrophs are simple and have been listed in previous chapters. Autotrophs are distinguished by their power to use inorganic carbon (CO_2). This is used wholly for synthesis and not for energy. In their energy sources they exhibit great variety and specificity. In this respect they are almost unique among living things. Some oxidize pure sulfur as energy food; others can use only NH_3, others CO, others nitrites, others CH_4, and so on. They will be described more in detail elsewhere.

Heterotrophs. Aside from carbon and energy sources, the *inorganic* requirements of heterotrophs appear to be similar to those of the autotrophs. Heterotrophs, however, are restricted to *organic sources of carbon* and gain their energy only from organic nitrogen or carbon sources, or both.

In both heterotrophs and autotrophs we know some of the uses to which certain elements in the inorganic foods are put. For example, iron and copper are used in certain enzymes; so are phosphate ions. Some metals serve as enzyme activators. Salts like sodium and potassium chloride maintain osmotic conditions. Carbon, hydrogen, oxygen and other elements are found everywhere in the cell structure in complex organic compounds. Some autotrophs oxidize S to H_2SO_4, NH_3 to $NaNO_3$, and so on. In the case of autotrophic nutrition we know the sources of each element and the material utilized for energy because we can furnish them in the foods and observe exactly what happens to them.

Organic Nutrient Requirements. Here we need deal only with heterotrophs, since autotrophs have no organic requirements. The organic requirements or heterotrophs vary greatly with different species. Some, as *Escherichia coli,* require only glucose (organic carbon) in an otherwise entirely inorganic medium. Others require a great number of organic compounds. The most generally used organic carbon sources are monosaccharides and disaccharides like glucose and lactose, and some polysaccharides like starch and dextrins. Simple organic acids and alcohols are also commonly available as carbon sources to heterotrophic microorganisms. Among the most readily used of these are lactic and succinic acids and similar compounds, as well as certain alcohols, as glycerol, etc. A great variety of related substances are used by certain organisms especially adapted to their utilization.

Organic nitrogen sources range from urea to 1 or more complex amino acids. In addition, various purines, pyrimidines, and like compounds may be desirable or necessary. Certain amino acids are more generally required than others, among these being glutamic acid, cystine, tryptophane, leucine or isoleucine and valine, but no generalization can be safely made as species differ in the number and kind of organic nitrogenous compounds required.

It is of interest to note that some essential compounds, as amino acids and vitamins, are of use to the organism because of the presence in those compounds of some particular molecular grouping that permits the *metabolite* or *nutrilite* (*i.e.,* essential food substance) to be used in a particular function, much as vitamins may function in coenzymes. If a compound similar to the metabolite, but differing in some slight degree, be furnished, it may combine within the cell just as the metabolite would do, but because of its somewhat different structure fails to carry on the physiological function of the essential metabolite so that the organism is injured, and dies or fails to grow. As seen elsewhere in this book, this is one of the basic ideas of chemotherapy (see page 226).

One of the difficulties in studies of heterotrophic nutrition is that of knowing which compounds are used in synthetic processes and which serve as sources of energy. With simple autotrophs, or species which will grow in synthetic media it is a relatively easy matter to determine these points by noting the products of oxidative reactions under various conditions or to substitute single sources of nitrogen, or carbon or energy in otherwise complete food mixtures of known composition and look for presence or absence of growth.

When using complex solutions like meat-infusion broth with peptone, one knows little about what serves for cell synthesis and what is used as a source of energy. Consider a culture of *Escherichia coli* in a tube of infusion broth to which lactose has been added. We know that lactose (as well as other materials) is metabolized, and changed into other products. Some of these can be found in the culture after growth but, in the words of Buchanan and Fulmer,[5] ". . . there is no simple means of determining whether the carbon assimilation [synthesis] needs are satisfied from the original compound [lactose] or from one of the products [such as lactic acid] . . ." Some organisms seem to use the same sorts of substance for both growth and energy but ". . . it is difficult to differentiate sharply . . . what proportion of organic carbon [as in lactose] may be utilized for energy and what assimilated.

"The energy derived [from the metabolism of organic compounds] may be utilized in part in evolution of . . . heat . . . light . . . movement, and in part may be stored as reserve food in the cell." Much light is now being thrown on these problems by the use of compounds containing radioactive isotopes of various elements.[6, 7] This is discussed farther on (page 280). It is significant in this connection that the actual *weights* of *bacterial substance* obtained with various compounds used as sole sources of carbon are often definitely related to the caloric or energy value of these compounds. Very careful and exhaustive studies by Werkman, Wood, Phelps, Johnson and Peterson and others indicate that, although heterotrophs do not use carbon dioxide as an exclusive source of carbon, carbon dioxide has an important place in the nutrition of heterotrophic bacteria. Many heterotrophic bacteria will grow much better in atmospheres containing from 2 to 10 per cent carbon dioxide.[*, 1] Most or all bacteria, both autotroph and heterotroph, may utilize carbon dioxide for cell synthesis.

It may be that CO_2 is the only form of C which some organisms can use for *synthesis,* or that synthesis proceeds with difficulty in its absence. At least, some organisms appear to require CO_2 (for synthesis) in addition to glucose (for energy), since they will not utilize glucose in the absence of CO_2.[8]

Photosynthesis and Chemosynthesis. We have spoken of the utilization by autotrophic bacteria of carbon dioxide and water to synthesize carbohydrates, etc., and it is well known that green plants accomplish the same thing. But whereas green plants require sunlight to perform this transformation, autotrophic bacteria (except certain photosynthetic species) achieve the synthesis in the absence of any light. In all forms of life it now appears likely that hydrogen is removed from water or, depending on the species, some other hydrogen-bearing compound (all of which are spoken of as *hydrogen donors*) and that the hydrogen is used in part to reduce carbon dioxide to some substance which is the basis of synthesis of various organic compounds.

Three important differences are seen between green plants and most bacteria in their mode of reducing carbon dioxide. These differences are manifest in

(*a*) *The source of energy used to extract the hydrogen* from the donor and transfer it to the carbon dioxide. In green plants energy is

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[*] A simple but effective method of cultivating bacteria in an atmosphere containing approximately 10 per cent CO_2 is to place the cultures in a jar containing a candle, light the candle and close the jar. The candle ceases to burn when the CO_2 content approaches 10 per cent.

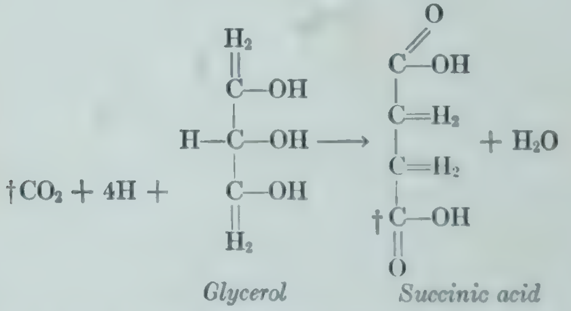
derived from sunlight which is absorbed and utilized in some manner by chlorophyll.* Cell synthesis utilizing CO_2 under the influence of light is called *photosynthesis.*

All bacteria (other than a few bacteriochlorophyll-bearing species are devoid of photosynthetic pigment and must obtain their energy for splitting H_2O and cell synthesis from chemical reactions involving oxidations. Cell synthesis based on this type of reaction is called *chemosynthesis.*

Chemosynthetic organisms, which include nearly all species of bacteria and all yeasts and molds grow well in the dark and are usually injured by direct sunlight. The mechanisms of photosynthesis in bacteria are discussed farther on in this book (Chapter 25).

(*b*) *The chemical nature of the hydrogen donor.* This may range from molecular hydrogen, through water and hydrogen sulfide, to organic compounds like CH_3COOH, and $C_8H_{15}COOH$, depending on the species of bacteria involved. Green plants use water exclusively, liberating oxygen. Certain photosynthetic bacteria utilize hydrogen sulfide, liberating sulfur.

(*c*) *Manner in which the carbon dioxide is assimilated.* In the case of green plants and strictly autotrophic bacteria, carbon-to-carbon linkages *must* be brought about directly between molecules originating from carbon dioxide. In heterotrophic species there is evidence that carbon from carbon dioxide molecules are not always linked directly together but that carbon from CO_2 may combine with already-formed organic molecules. For example, Werkman, Carson, Wood, and their colleagues have "fed" carbon dioxide made with "heavy" carbon (C^{13}) and radioactive carbon (C^{11}) to propionic acid bacteria. The special carbon can be followed, like a tracer bullet, through its course in the synthetic processes of the bacterial cell. For each molecule of the radioactive carbon dioxide, one molecule of succinic acid or propionic acid is formed by the organism from a molecule of glycerol.



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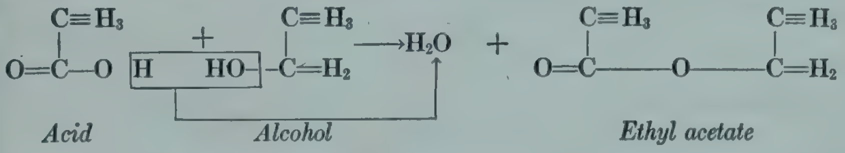
* In the case of a few species of photosynthetic bacteria, sunlight is utilized by a closely related compound called *bacteriochlorophyll* (see Chapter 25).

† *Radioactive C.*

The use of such "tagged" atoms of various elements is providing one of the most useful tools for elucidation of problems of metabolism, including those surrounding the utilization of carbon.[9] This in turn will one day enable us to understand how green plants perform, under our very noses, one of the great mysteries of life—the utilization of carbon dioxide and water to form such material as sugar, starch, wood, etc. Many other problems, such as the metabolism of cancer tissues, may yield to this method of study.[10, 11, 12]

Assimilative Reactions. In cell synthesis from complex organic materials certain of the atoms and molecules into which the food substance is first divided by hydrolytic and other enzymes as described in the last chapter, are rearranged, combined with various minerals, etc., and put together again in new structures constituting the protoplasm, cellulose, fat, glycogen, chitin, volutin, and other portions of the organism. The reactions involved are synthetic and are *endothermic* since energy is absorbed and stored in the complex molecules resulting from the synthesis or building process. Many of the endothermic reactions are the reverse of hydrolysis, sometimes called polymerization or condensation. When the endothermic reaction results in the synthesis of actual cell substance, *assimilation* is said to have occurred.

As pointed out in the section on enzyme action, some hydrolytic changes are reversible, and complex molecules are in part built up from simpler ones by the abstraction of a molecule of water. It is not unlikely that in some instances the same enzymes bring about the changes in either direction, the conditions in the cell determining the direction of the reaction. A simple type of synthetic reaction is seen in the formation of ethyl acetate from acetic acid and alcohol:



$$O{=}\overset{C\equiv H_3}{\overset{|}{C}}{-}O\ \boxed{H \quad + \quad HO}{-}\overset{C\equiv H_3}{\overset{|}{C}}{=}H_2 \longrightarrow H_2O + O{=}\overset{C\equiv H_3}{\overset{|}{C}}{-}O{-}\overset{C\equiv H_3}{\overset{|}{C}}{=}H_2$$

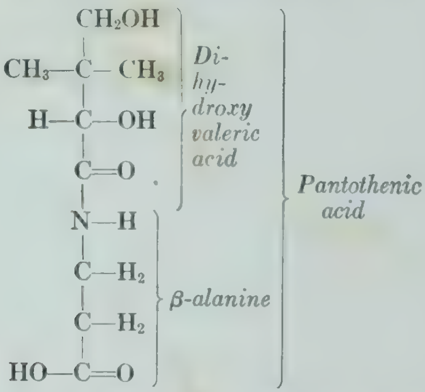
or in the building of polypeptides from amino acids (see page 282). Reactions resulting in the decomposition of food and liberation of energy are discussed in the next chapter.

Growth Factors. Growth factors, essential *metabolites* or *nutrilites,* are substances which do not yield energy to the cell or contribute materially to its bulk, but which are absolutely essential to its continued well-being. Vitamins are good examples of growth factors, as well as certain metallic elements. Certain specific amino acids have the status of growth factors; that is, they are among the substances absolutely essential to the growth of a number of bacteria. A good example is tryptophane, without which no growth of the typhoid bacillus (*Sal-*

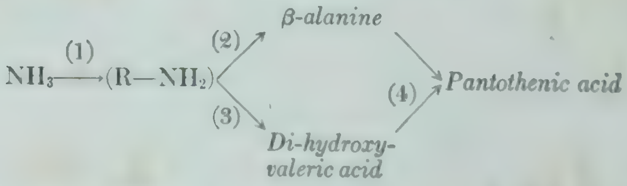
monella typhi), the lockjaw organism (*Clostridium tetani*), the diphtheria bacillus (*Corynebacterium diphtheriae*) and several others can occur, even though their culture medium be complete in all other respects. On the other hand, there are many bacteria which can synthesize their own tryptophane and so do not need to have it fed to them. Among these are autotrophic bacteria, *Pseudomonas aeruginosa* (a soil and water saprophyte) and numerous others which, though grown in a tryptophane-free medium, nevertheless synthesize this amino acid.

It is probable that such critical amino acids play a role in the building of protoplasm analogous to that of vitamins, *i.e.*, they are a part of a single vital component in the cell. A number of other critical substances or growth factors are known or are under investigation. Among them are nicotinic acid, riboflavin, thiamine, pantothenic acid and other vitamins.[13, 14]

A probable role of such compounds or growth factors in bacterial nutrition was described by Fildes.[15] Assume that an organism requiring pantothenic acid, a vitamin, is unable to synthesize β-alanine (one of the amino-acid radicles in, or associated with, pantothenic acid),



but is able to carry out all other syntheses, beginning with simple ammonia, necessary to the formation of pantothenic acid (see 1, 3 and 4 in the diagram below). Given a minimum quantity of β-alanine completion of the pantothenic acid is provided for and growth occurs Without β-alanine, no growth is possible.[16-25]



A strain or species able to synthesize β-alanine as well as the othe components of pantothenic acid encounters no such difficulty an we say that "it does not require β-alanine." Both may require β-alanine, but one can manufacture it internally, the other cannot.

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Chapter 15

THE METABOLISM OF MICROORGANISMS

2. Dissimilation; Oxidation

In the chapter on enzymes we reviewed the mechanisms by which microorganisms bring about the chemical changes that are manifested as life; and in the chapter on nutritive requirements we have inspected and discussed the larder and pantry, of microorganisms, *i.e.,* their foodstuffs, and how they are assimilated into the cell substance. In this chapter we shall see how the organisms use the foods as energy sources, *i.e.,* the processes of dissimilation and respiration or biological oxidation.

The chemical reactions of dissimilation and respiration yield energy or are *exothermic*. In dissimilation, as a rule, complex molecules are broken down (degraded) to simpler substances in a series of stepwise chemical reactions and the energy, supplied by some other organism to synthesize the complex substance, is liberated slowly, in steps, by the organism using that substance as a source of energy.[1, 2, 3]

DISSIMILATION

An important preliminary to the use of many complex molecules as food is the process of "digestion," *i.e.,* hydrolysis. We should, therefore, say a few words about this process before discussing the exothermic reactions.

BACTERIAL DIGESTIVE PROCESSES

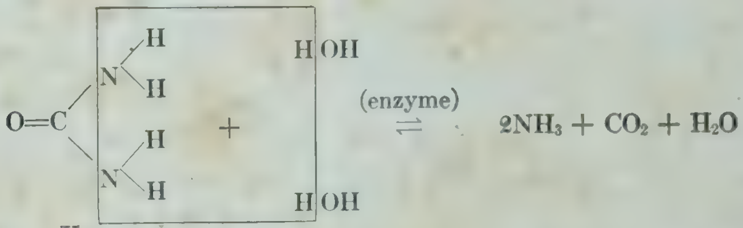
Bacteria, it has been pointed out, cannot take through their cell membranes any solid matter, or even any very large molecules. Yet they are capable of utilizing as food dead leaves, the trunks of fallen trees and other plant matter, as well as crab shells, hair, horn, flesh of dead animals and other organic debris. The agents by means of which they accomplish these wonders are enzymes, notably *carbohydrases, esterases* and *proteinases,* which are *hydrolytic* in their action. These are exoenzymes, acting outside the cell.

These bring about the decomposition of large, complex molecules into smaller ones, *soluble in water* and capable of diffusing through the cell membrane so as to be acted upon as food by the intracellular catalysts. In this way, by extracorporeal digestion as it were, bacteria can utilize large masses of solid matter as food. Since these hydrolytic decompositions occur outside the cell, any energy released is lost as far as its usefulness to the cell is concerned. Reactions which yield energy to the cell must obviously occur within or on the cell.

Examples of hydrolytic reactions are the splitting of cane sugar into *d*-glucose and *d*-fructose:

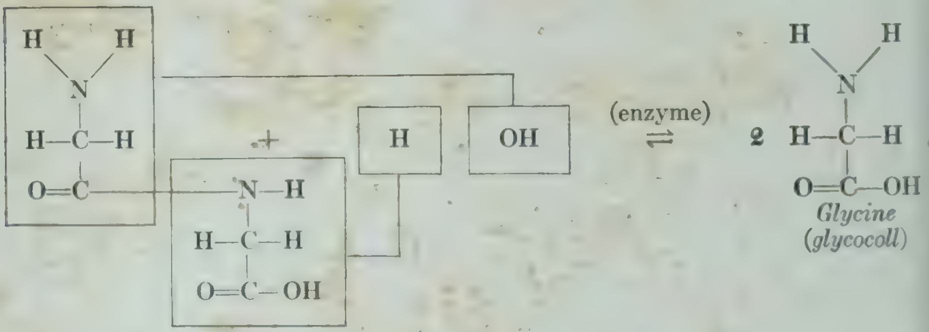
$$\underset{\textit{Saccharose}}{C_{12}H_{22}O_{11}} + H_2O \overset{\text{(enzyme)}}{\rightleftharpoons} \underset{\textit{d-Glucose}}{C_6H_{12}O_6} + \underset{\textit{d-Fructose}}{C_6H_{12}O_6}$$

or the breaking-up of urea into ammonia and carbon dioxide:



Urea

or the division of 1 molecule of the polypeptid glycylglycine into two of glycine:



Glycylglycine

Note that a molecule of water is introduced into the molecule of substrate in all hydrolytic reactions. Note also that the reactions are reversible and that synthesis or assimilation may in some cases proceed by the reverse of the proces of hydrolysis.

Carbohydrate Hydrolysis. Excellent examples of hydrolysis are found in the scavenging of various organic wastes in the soil, for example, in the decomposition of cellulose and other plant carbohydrates.

Consider the trunks of enormous trees that fall; the acres of leaves; mountains of vegetable wastes from canneries, and the tons of cornstalks, straw, stubble and vegetable matter which are plowed into the soil. What happens to all of these? The material composing the rigid, woody parts of plants as well as the skeletal structure of soft parts is principally cellulose, or a dense material of the same nature called "lignocellulose," combined in various ways with gums, starch, waxes, pectin, and the like. The ligno-cellulose and cellulose are complex carbohydrate materials chemically related to starch; and made up of glucose molecules, *i.e.,* they are polysaccharides. Wood, of course, is relatively resistant to water, weather, cold, weak acids, alkalies, and other influences and will withstand them for considerable periods, sometimes years. However, soil microorganisms decompose most wood rapidly.

Many kinds of molds and larger fungi produce enzymes which *hydrolyze* ligno-cellulose and cellulose into molecules of simpler carbohydrates like hemi-cellulose and also into cellobiose. Many bacteria can hydrolyze cellulose to cellobiose. Cellobiose, in turn, is hydrolyzed by a considerable number of bacteria and molds into simple molecules of glucose. Still other organisms (or perhaps the same ones) attack the starches, etc., in various plant structures and hydrolyze them, producing, in the case of starch, successively erythrodextrin, achrodextrin, dextrins, maltose and finally glucose.* Glucose is a principal source of energy for practically all organisms.

In the decomposition of wood and cellulose in the soil, each successive and simpler derivative serves as a source of carbon or energy, or both, for organisms which can decompose that derivative.

The final products of the decomposition depend on the sorts of bacteria and molds present in the particular soil under consideration and on whether abundant oxygen is present or not. If air is present in abundance, highly oxidized substances such as carbon dioxide and water are generally the chief end-products, with "humus" remaining as a soft, brown substance so desirable in fertile soils and slowly undergoing the last stages of the decay process. In case abundant air is not present (as in the muck of boggy or swampy places or heavy clay soils), more "intermolecular" and "intramolecular respiration" or *fermentation* (see below) is likely to occur, with the development of reduced and partly oxidized end-products such as methane, hydrogen, alcohols, and butyric and other organic acids which contribute to the acidity of "sour" soils.†

The hydrolytic decomposition, in the soil, of a complex substance such as wood somewhat resembles the dismantling of a large mansion. First, doors, window-sashes, stair-rails, fireplace mantels, and so on, are removed. Then whole sections of roof, wall or flooring may be taken down. (All these parts would correspond to rather large molecules like cellobiose and various dextrins into which the cellulose, starch and other polysaccharides are at first split.) It may be that neighboring builders can utilize these same doors, window frames, stairs and roof sections intact. This would correspond to the utilization by various bacteria of the split compounds—dextrins, cellobiose and others—resulting from the progressive splitting up of the woody material. Other smaller and simpler constructors may require that the larger sections of the house be broken down to their constituent boards and bricks before making use of them. This would correspond to the final hydrolysis to glucose, of which nearly all the other carbohydrate molecules are built, and its utilization by being further split and metabolized by still other bacteria. We shall see later the uses to which the glucose is put by various bacteria.

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* A similar process can be brought about by boiling wood, corn cobs and similar substances with acid for long periods. (See section on industrial alcohol production, page 679.)

† The draining and liming of such soils is necessary for agriculture since most of the bacteria (especially the genera *Nitrobacter, Nitrosomonas* and *Nitrosococcus,* and the genera *Azotobacter* and *Rhizobium,* all of which will be discussed) are sensitive to acid and are aerobic.

Protein Hydrolysis. Protein is built up of amino acid molecules much as complex carbohydrates are made up of glucose molecules. They are linked in the manner shown for the combination of glycine units in glycylglycine (see page 286). Amino acids are the "building stones" of protein or protoplasm. Hydrolytic enzymes (proteinases) first split proteins into large "sections" which are, chemically, albumoses, proteoses, and polypeptides of various degrees of complexity (glycylglycine is a simple polypeptide, a dipeptide). This hydrolysis is analogous to the splitting of wood into hemi-cellulose, cellobiose, and glucose, or starch into dextrins and so on. The larger nitrogenous molecules (albumoses, etc.), are further and further decomposed by hydrolysis until finally the amino acid stage is reached. Hair, horn, hoof, insect and crustacean shells (chitin), flesh, all of which are protein or compounds related to protein, are similarly decomposed, primarily by hydrolysis, into simpler substances available as food.

This hydrolysis, similar to that of complex carbohydrates, further resembles it in that the end-products of complete hydrolysis are relatively simple units—glucose in the case of carbohydrates, amino acids and related compounds in the case of proteins. In either case the cell deals most directly with the glucose or amino acids. We shall discuss later the uses to which the end-products of protein hydrolysis are put.

The fats, waxes, and other complex or insoluble materials are similarly reduced to simpler substances of smaller molecular size by processes of hydrolysis.

The digestive processes just described are essential to the metabolic utilization of complex organic compounds like wood and protein by heterotrophs, but what of the autotrophs? These use simple minerals or elements as energy sources and need not hydrolyze them first. Thus we arrive at three basic types of food and energy sources: glucose, exemplifying a large class of relatively simple non-nitrogenous organic compounds, including hexoses, alcohols, esters, etc.; amino acids, illustrative of a great group of relatively simple organic nitrogenous substances, including urea, purines, etc.; and inorganic substances such as elemental sulfur and hydrogen, carbon monoxide, carbon dioxide, ammonia, nitrites, sulfides, etc.

RESPIRATION OR BIOLOGICAL OXIDATION

The term *respiration* is frequently used interchangeably with biological oxidation because in animals atmospheric oxygen is inhaled by means of the respiratory apparatus (breathing) and was thought of as combining with foodstuffs which are thus "burned" or oxidized in the body. Later, as a result of Pasteur's studies on fermentation, it became evident that such respiratory functions are not the only means by which living beings obtain energy, yet the term respiration continues to be used to signify any exothermic reactions having an energy value for living organisms, whether free oxygen and a respiratory apparatus like lungs were involved or not. In speaking of respiration it has become customary to use the term biological oxidation, and its chemical connotation in the newer sense is quite broad and includes both aerobic and anaerobic respiration.

Fundamentally, oxidation means the loss of electrons, as by change of valence, *e.g.*,

$$\mathrm{Fe}{++} \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftarrows}} \mathrm{Fe}{+++} + \epsilon.$$

Reduction, conversely, implies the gain of electrons. For example, silver in solution (*i.e.*, in the form of Ag^+ ions) is not in an obviously oxidized form. Yet, to demonstrate its oxidized condition, it is necessary only to *reduce* the ion Ag^+ with formaldehyde or glucose, *e.g.*, add electrons to it, to change it to un-ionized, metallic Ag and have it precipitate on a glass surface as is done industrially to make mirrors. It is important to note that in bringing about the reduction of the silver some other substance, which gives up its electrons, becomes oxidized. This compensatory give and take is also characteristic of biological respiratory reactions. An oxidation of one thing implies a reduction of something else, and vice versa. As oxidation or electron transfer is always going on during life, living creatures maintain a definite *oxidation-reduction electrical potential* in the cell and in their immediate environment, the reducing intensity of which depends on many factors such as the species, its age, variation phase, and the environment. Since oxidative reactions always involve electron transfers we are able to study them by means of instruments capable of measuring electrical potentials (potentiometers).

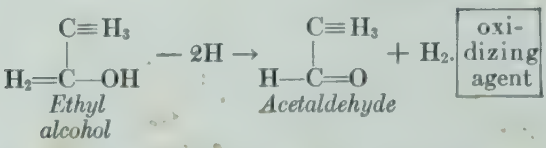
Biological Oxidation Reactions. We may here consider several kinds of reactions which may be regarded as typifying biologically significant oxidations. These reactions are brought about by respiratory enzymes which are discussed farther on.

1. The loss of an electron (change of valence).

This is in accord with the facts outlined above. The oxidizing agent is here serving as an electron acceptor. Any transfer of electrons accomplishes both oxidation and reduction since the electron is finally attached to some appropriate acceptor which becomes reduced. The electron cannot remain free.

2. The loss of hydrogen.

$$H_2S - 2H \longrightarrow S + H_2. \boxed{\text{oxi-dizing agent}}$$



$$H_2{=}\overset{\overset{\displaystyle C\equiv H_3}{|}}{C}{-}OH - 2H \rightarrow H{-}\overset{\overset{\displaystyle C\equiv H_3}{|}}{C}{=}O + H_2. \boxed{\text{oxi-dizing agent}}$$

Ethyl alcohol *Acetaldehyde*

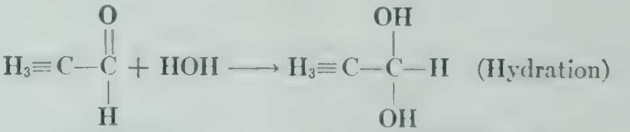
Here the hydrogen combines with some hydrogen acceptor (oxidizing agent) such as atmospheric oxygen. Other reducible substance like sodium nitrate, sulfates, sulfur, etc., may serve. These latter mechanisms are found in anaerobic metabolism. As in example 1, an electron is transferred (at the time hydrogen is transferred) so that an energy transfer is involved. As will be seen later, hydrogen transfer is one of the most important means of biological oxidation.

3. The addition of oxygen.

$$2H + \tfrac{1}{2}O_2 \longrightarrow H_2O$$
$$CO + \tfrac{1}{2}O_2 \longrightarrow CO_2$$

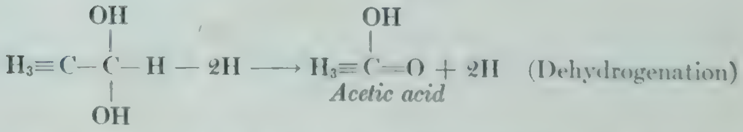
In this sort of respiration free atmospheric oxygen usually serves as the oxidizing substance, and such reactions are characteristic of aerobic respiration. More than half of aerobic respiration is by this means.

4. Hydration and dehydrogenation.



$$H_3{\equiv}C{-}\underset{\underset{\displaystyle H}{|}}{\overset{\overset{\displaystyle O}{\|}}{C}} + HOH \longrightarrow H_3{\equiv}C{-}\underset{\underset{\displaystyle OH}{|}}{\overset{\overset{\displaystyle OH}{|}}{C}}{-}H \quad \text{(Hydration)}$$

Acetaldehyde *Acetaldehyde hydrate*



$$H_3{\equiv}C{-}\underset{\underset{\displaystyle OH}{|}}{\overset{\overset{\displaystyle OH}{|}}{C}}{-}H - 2H \longrightarrow H_3{\equiv}\overset{\overset{\displaystyle OH}{|}}{C}{=}O + 2H \quad \text{(Dehydrogenation)}$$

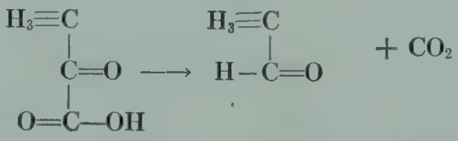
Acetic acid

Reactions of type 4 are really a combination of the types of reaction given in 2 and 3.

Various forms of these basic types of reaction will be illustrated later on.

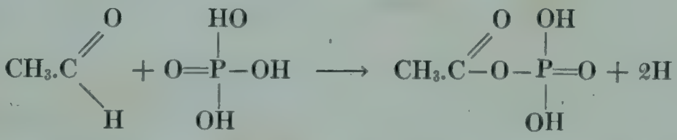
Several other exothermic reactions are known to occur, and there may be many unknown energy-yielding transformations. Not all can be discussed here, but three more may be mentioned, *decarboxylation, phosphorylation* and *molecular rearrangement.*

5. *Decarboxylation* results in splitting the carboxyl group of organic acids, such as pyruvic, thus:



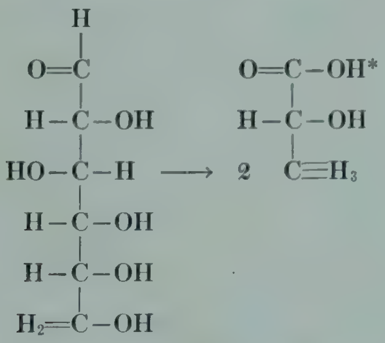
$$\begin{array}{c} H_3{\equiv}C \\ | \\ C{=}O \\ | \\ O{=}C{-}OH \end{array} \longrightarrow \begin{array}{c} H_3{\equiv}C \\ | \\ H{-}C{=}O \\ \\ \\ \end{array} + CO_2$$

6. *Phosphorylation* is an oxidative process involving the combination of inorganic orthophosphate to organic compounds with double bonds accompanied by dehydrogenation, thus:



or the phosphate may be combined with carbohydrates incident to their dissimilation. Energy is liberated when the phosphate is released from the compound.

7. *Molecular rearrangement.* This results in a transfer of hydrogen in a molecule so that one part is reduced and the other oxidized simultaneously, the net result being a release of energy, thus:



$$\begin{array}{c} H \\ | \\ O{=}C \\ | \\ H{-}C{-}OH \\ | \\ HO{-}C{-}H \\ | \\ H{-}C{-}OH \\ | \\ H{-}C{-}OH \\ | \\ H_2{=}C{-}OH \end{array} \longrightarrow 2 \begin{array}{c} O{=}C{-}OH^{*} \\ | \\ H{-}C{-}OH \\ | \\ C{\equiv}H_3 \end{array}$$

Glucose *2 lactic acid*

MECHANISMS UNDERLYING BIOLOGICAL OXIDATIONS

The physiological systems which carry the various types of exothermic reaction to completion are complex and include the respiratory enzymes and several coadjutant bodies. The enzymes themselves, as previously stated, generally consist of a colloidal protein, which may or may not have the property of specificity, combined more or less firmly with a less complex, nonprotein, noncolloidal body called a prosthetic group or coenzyme which generally has the property of specificity.

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* This reaction probably proceeds in two or three stages.

About the chemistry of the protein moiety of these enzymes there is not much information which is pertinent to our present discussion. More is known of the coenzymes or prosthetic groups. Several of them have been artificially synthesized. We may here discuss (1) the main functions and properties of the respiratory enzymes as complete structures; (2) the nature, function and composition of several typical coenzymes and other substances having similar functions but which are not so closely related to enzymes; and (3) their relation to certain of the vitamins.

Respiratory Enzymes. These are true endoenzymes or *desmolases* and the energy they release is used inside the cell. Important respiratory enzymes are *dehydrogenases, oxidases, peroxidases, mutases,* and *phosphorylases.*[4, 5, 6]

1. *Dehydrogenases.* These cause hydrogen to leave the substrate. All dehydrogenase systems involve a *hydrogen donor* (the substrate being oxidized) and a *hydrogen acceptor* (a substance being correspondingly reduced) in order to complete the exothermic transfer of hydrogen. There are two kinds of dehydrogenase systems, which differ in respect to the acceptor of hydrogen. These are the aerobic and the anaerobic dehydrogenases. These operate by means of specific coenzymes.

Aerobic dehydrogenases activate* the hydrogen of the substrate and cause it to combine either directly with molecular oxygen, forming water or hydrogen peroxide, or with some other readily reducible compound such as sulfur, forming hydrogen sulfide, or sodium nitrate, forming sodium nitrite and water.

Anaerobic dehydrogenase systems do not make use of molecular oxygen directly, but they transfer hydrogen to an intermediary "respiratory middleman" called a *hydrogen carrier.* This accepts hydrogen from the substrate (in the presence of the dehydrogenase) and transports it toward a final acceptor, which may be molecular oxygen, carbon, sulfur, nitrogen, or other reducible substances, often through the intermediation of a second carrier or several successive carriers. Each carrier has the property of accepting hydrogen from a more reduced compound in the hydrogen transfer system and of being itself in turn dehydrogenated by a component in a less reduced state. These linked systems of hydrogen transfer have been referred to as "physiological bucket brigades." Anaerobic dehydrogenases never produce H_2O_2, but typically produce H_2S, NH_3, CH_4, etc.

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* The exact meaning of this word is not entirely clear but we may think of i as indicating an induced instability of hydrogen so that it readily leaves the molecule of which it is a part and combines with some other substance; or as an instability of any substance so that it becomes chemically active. The explanation is a major problem in biochemistry. The term "mobilization" is sometimes used in place of activation.

Diaphorase or Coenzyme Factor. This is an important and widely distributed enzyme, apparently a true dehydrogenase. It seems to catalyze the removal of hydrogen from the reduced prosthetic group of dehydrogenases and the transfer of the hydrogen to the next carrier, often a respiratory pigment (cytochrome) analogous to the red pigment of erythrocytes.

Note that dehydrogenases are inactivated by alcohol, urethane and some narcotics.

2. *Oxidases.* These may either activate molecular oxygen as hydrogen acceptor, the active oxygen being reduced to water or hydrogen peroxide; or they may catalyze a combination of molecular oxygen directly with some substrate, *e.g.*, reduced cytochrome. In either case the oxygen combines with hydrogen. Two outstanding properties of oxidases are that (*a*) their activity is inhibited by low concentrations of carbon monoxide, potassium cyanide and hydrogen sulfide; and (*b*) they can act only in the presence of *free* oxygen, that is, they are obligately aerobic. Coenzymes are essential in their action.

Examples of oxidases are (*a*) cytochrome oxidase (indophenol oxidase) which is very common and is found with cytochrome in virtually all aerobic cells; and (*b*) ascorbic acid (vitamin C) oxidase. The latter plays an important role in the physiological activity of vitamin C. There also are amino-acid oxidases and several others.

3. *Peroxidases.* These either decompose the hydrogen peroxide formed by oxidases liberating free oxygen to the air (catalase), or decompose the hydrogen peroxide but, unlike catalase, activate the oxygen so that it combines with readily oxidizable substrates. For example, $NaNO_2$ may be oxidized to $NaNO_3$. Outstanding properties of peroxidases are (*a*) their oxidative function, like that of oxidases, is inhibited by carbon monoxide, hydrogen sulfide and potassium cyanide; and (*b*) they often act in *very low* concentrations of hydrogen peroxide so that lethal hydrogen peroxide accumulation due to the activity of oxidases does not occur. (See section on anaerobiosis, page 305.)

4. *Mutases.* These are enzymes which, as the name implies, catalyze changes in form (dismutations) of certain molecules, reducing one part of the molecule and oxidizing another part, or reducing one molecule of a given substance and oxidizing another, usually splitting a water molecule between the two—a sort of hydrolytic reaction, called by the name of its discoverer Cannizzaro.

5. *Phosphorylases.* These enzymes are involved in the transfer of

energy-rich phosphate bonds to organic compounds. They take up the phosphate from one compound and give it up to another, much as hydrogen is transferred from one substance to another. Like the hydrogen transport and other respiratory systems, the phosphate transfers are carried on by the help of coenzymes. The splitting of the phosphate bonds liberates much energy, and the formation of organic phosphates is a means of storing energy derived from oxidative processes in the cell.

Coenzymes. As pointed out previously, enzymes appear to consist of two components, a protein and another group which may be loosely or firmly bound to the protein body. Where separation has been possible, the nonprotein components have been found to have certain physical properties in common. In general they differ from complete enzymes, or the colloidal protein portions of them, in that they are heat-stable, water-soluble, and dialyzable (*i.e.,* they are smaller molecules than enzymes). They are complex organic compounds and are active participants in enzyme reactions but they are not complete enzymes. Some can be synthesized. They are closely related to, or contain, vitamins. These nonprotein portions of enzymes are called *coenzymes*. They are also known as the *prosthetic group* of their respective enzymes.

We have just seen that respiratory enzymes may be classed on a basis of their functions and other properties. Respiratory enzymes may also be conveniently classified according to the chemical nature of their prosthetic groups. Here we shall discuss five different prosthetic groups, each part of a specific respiratory enzyme. These prosthetic groups or coenzymes are (1) the pyridine nucleotides (codehydrogenases and co-mutases); (2) thiamine pyrophosphate (cocarboxylases); (3) alloxazine-adenine-dinucleotide (co-oxidases); (4) adenylic acid compounds (co-phosphorylases).

1. *The Pyridine Nucleotides.* (*Coenzymes I and II,* or diphosphopyridine nucleotide and triphosphopyridine nucleotide, respectively.) These coenzymes are readily separable from the protein component of their enzyme complex by washing or dialysis. They accept hydrogen from substrates whose hydrogen is activated by dehydrogenases, later giving it up to some other substance. Specificity appears vested in the coenzyme and not in the protein moiety. Their composition (Fig. 129) is known from the fact that on hydrolysis they yield:

1 mol nicotinic acid amide (hydrogen-combining radicle),
1 mol adenine,
2 mols pentose,
2 or 3 mols phosphoric acid.

They are readily reoxidized (dehydrogenated) by oxygen or other substances which take their hydrogen from them and which thus carry forward the respiratory hydrogen transportation initiated by the enzyme-coenzyme combine.

It is of interest to note that some bacteria, *e.g., Staphylococcus aureus, Lactobacillus arabinosus,* various strains of *Salmonella,* etc., are able to synthesize part of coenzyme I but must have the nicotinic acid amide, which is a part of it, given them in a preformed condition in order to complete the process. Nicotinic acid, nicotin amide, or other isomers may or may not serve, depending on the synthetic powers of the species; *H. influenzae* seems to require the entire coenzyme molecule in a preformed state and cannot use nicotinic acid to manufacture it.

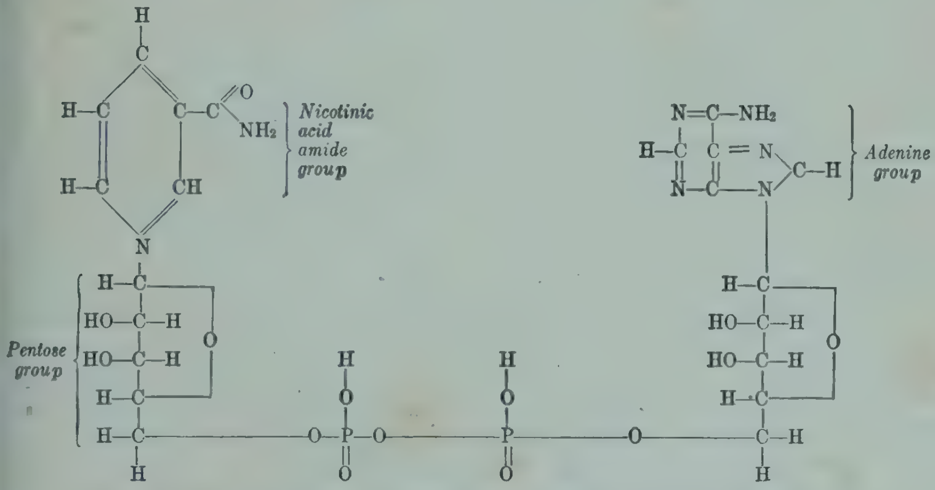
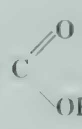


Fig. 129. Coenzyme I. This is attached to a protein body.

In human beings affected with pellagra (a disease due to nicotinic acid deficiency), administration of nicotinic acid alone is sufficient to produce a marked increase in the amount of *coenzyme* in the blood and to allay the symptoms of the disease, so that the role of nicotinic acid in human metabolism seems fairly indicated as a chemically active radicle or part of the coenzyme molecule in cell respiration. Dysentery bacilli apparently do not metabolize glucose normally in the absence of nicotinic acid, and seem to be injured by the sugar in its absence. Indeed, the vitamin appears to play a decisive role in carbohydrate metabolism by most cells and the reason appears to be its critical importance as a part of the respiratory coenzymes I and II, since carbohydrates serve as sources of energy through the action of respiratory enzyme systems.[7–10]

2. *Thiamine Pyrophosphate (Cocarboxylase).* This coenzyme is associated with the enzyme carboxylase of various cells (yeast, bac-

teria, etc.) in the decomposition of the carboxyl group  $C\genfrac{}{}{0pt}{}{\diagup O}{\diagdown OH}$ especially of pyruvic acid.

Cocarboxylase is widely distributed in animals, yeast, vegetables, bacteria, etc. It has been artificially synthesized and consists of thiamine linked with two phosphate groups (Fig. 130). It shows vitamin B_1 (thiamine) activity and maintains normal carbohydrate metabolism in animals. This is because decarboxylation of pyruvic acid is an essential step in the metabolism of glucose by many types of cell. For example, if pyruvic acid accumulates in the blood of human beings we have the disease known as beriberi. In thiamine deficiency of animals, the pyruvates accumulate in the blood, causing similar serious disturbances.

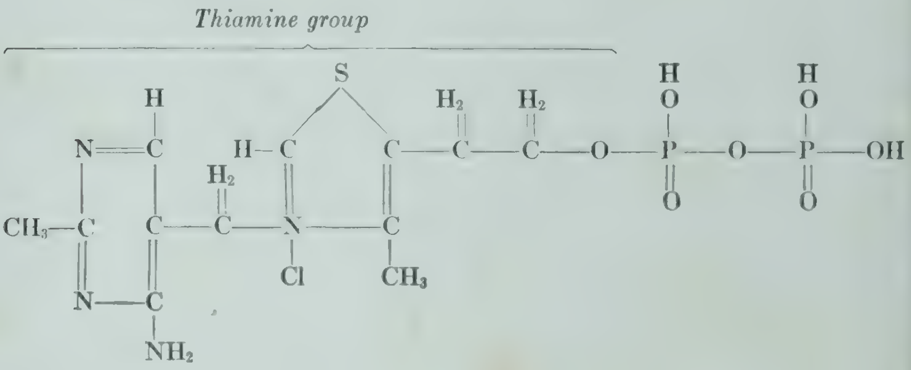


Fig. 130. Cocarboxylase.

In studies of bacteria, pyruvic acid and acetaldehyde have been shown to occur during glucose fermentation and the function of cocarboxylase may tentatively be inferred as essential in the decarboxylation of the acid, producing the aldehyde. Some species, *e.g.*, *E. coli* and acid-fast bacteria, can apparently synthesize cocarboxylase from inorganic substances with glucose; others, *e.g.*, species of *Propionibacter*, require thiamine preformed in their medium.[11-14]

3. *Adenine-alloxazine-dinucleotide (the Coenzyme of d-Amino Acid Oxidase, or the Deaminase Coenzyme.* This coenzyme is associated with the enzyme *d*-amino acid oxidase to catalyze the deaminative oxidation of *d*-alanine to pyruvic acid by molecular oxygen.

$$\underset{\textit{d-alanine}}{CH_3.CH.NH_2.COOH} + \tfrac{1}{2}O_2 + H_2O \longrightarrow \underset{\textit{Pyruvic acid}}{CH_3.CO.COOH} + NH_4OH$$

The two components of the enzyme are easily separated. Like cocarboxylase, deaminase coenzyme is widely distributed in animals and plants.

The *d*-amino acid oxidase is one of a number of enzymes having coenzymes containing flavin in the coenzyme (see Fig. 131). Not all are oxidases. One of these is diaphorase of coenzyme factor. As a group they are often referred to as "yellow enzymes" because of the yellow color of the flavin compounds.

It is of interest to compare the formula of *d*-amino acid oxidase coenzyme (Fig. 131) with that of coenzyme I. The two are similar except in the tricyclic form of one radicle, and the additional nitrogen content. The tricyclic group (alloxazine), together with the ribose group complete, constitutes the yellow-colored flavin grouping called riboflavin, or vitamin B_2 (or G).

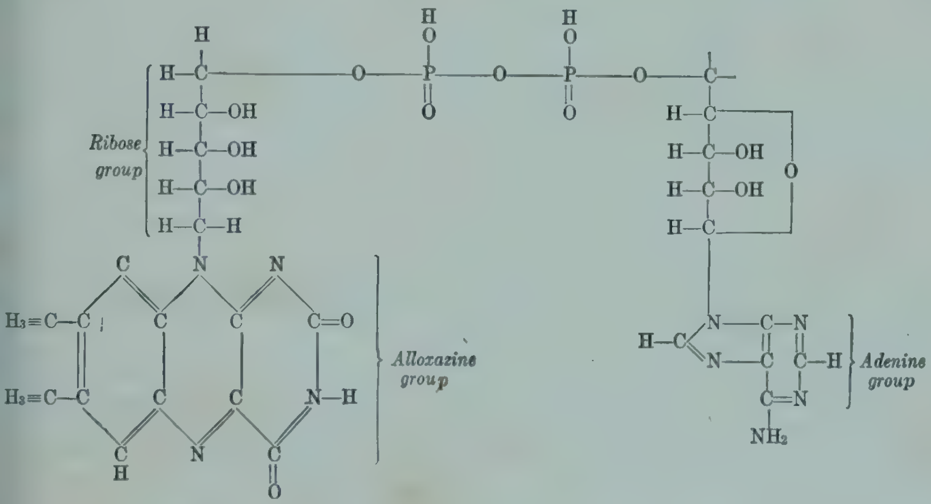


Fig. 131. Coenzyme of the *d*-amino acid oxidase.

The yellow respiratory enzymes are involved in the final oxidations of hydrogen, cytochrome, or reduced coenzymes I and II by molecular oxygen.

Other Respiratory Agents. *1. Metallo-pigments.* The yellow flavin-enzymes, while pigments of a sort since they are yellow in color, do not contain heavy metal atoms. The metallo-pigments best known in respiration are (1) *hematin,* the iron-containing pigment of the red blood cells; (2) *chlorophyll,* the pigment of all green plants, which contains magnesium; (3) the group of *hemocyanins,* found in shellfish and consisting of copper-protein complexes; (4) *bacteriochlorophyll,* occurring in photosynthetic bacteria and acting like chlorophyll; and (5) various *cytochromes* (a, b and c). The last contains iron which, in the respiratory process changes its valence from 2 to 3 and back.

The cytochromes occur in bacteria yeasts, etc., and the intensity of aerobic respiration is related to the amount of cytochrome (mainly C) present. Cytochrome accepts hydrogen from a substrate, for example, reduced coenzyme, and the final oxidation of the hydrogen by molecular oxygen is then catalyzed by the equally widely distributed enzyme, cytochrome oxidase, with the formation of water.

Cytochrome is involved in from one half to two thirds of the aerobic respiration of many bacteria.

2. *Glutathione.* This is a heat-stable compound, the biologically significant part of which is the sulfhydryl (—SH) grouping (Fig. 132), since this gives up or acquires hydrogen readily, thus playing a respiratory role.

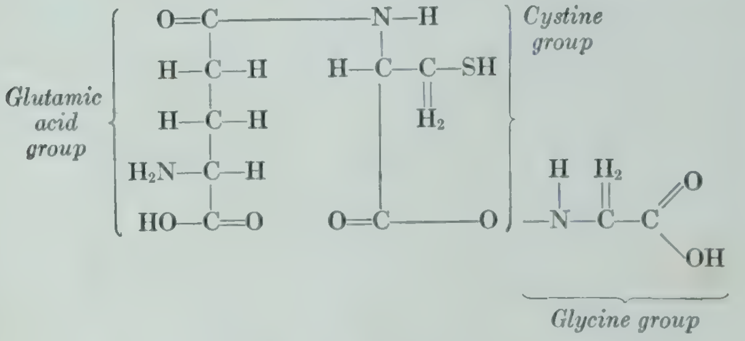


Fig. 132. Glutathione.

Glutathione is widely distributed in animal tissues and in bacteria. Its exact role in bacterial respiration has not been clearly demonstrated but it may act as a carrier, for example, in the formation of lactic acid from pyruvic acid (see schema on page 300). It contains the cystine radicle with its —SH grouping. In the presence of iron compounds like cytochrome it is rapidly oxidized (dehydrogenated) by molecular oxygen. Thus, a hydrogen donor (R·H) gives its hydrogen to glutathione (which we may represent by the symbol GS), leaving R oxidized and producing GSH. If pyruvic acid acts as hydro-

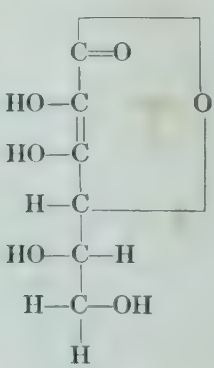


Fig. 133. Ascorbic acid.

gen acceptor from glutathione, the pyruvic acid is changed into lactic acid and GSH becomes GS again. Glutathione probably also plays some role in preventing excessive and destructive oxidation of ascorbic acid (when this is present) and probably other related compounds, since it has a definite reducing action as it exists in the cell.

3. Ascorbic Acid (Vitamin C). Vitamin C is chemically relatively simple (Fig. 133). Its exact role in respiration is not clear. Its antiscorbutic properties and probably other functions, such as protection of complement, in the blood are doubtless related to its chemical reducing property. It is destroyed by excessive oxidation. Ascorbic acid may act as a hydrogen acceptor in the oxidation of glutathione.

STAGES IN BIOLOGICAL OXIDATIONS

Having reviewed the basic reactions and the mechanisms involved in biological respiration we may now see how these systems act in the final stages of decomposition of food substances resulting in the liberation of energy. The most instructive example is the degradation of glucose.[15, 16, 17]

Dissimilation of Glucose. As we have seen, glucose (or other carbohydrates) may serve both as an energy source and as a source of carbon for cell synthesis. It is difficult to determine just how the organisms arrange these matters. However, we can survey by chemical means the "left-overs," after the organism has metabolized the carbohydrate, and we may add chemical indicators or traps of various sorts to the growing cultures to detect substances which, although not appearing among the end-products, may be formed temporarily during the dissimilative or oxidative process. Several of the intermediate products in glucose dissimilation have been isolated or identified by precipitation or colorimetric methods. Another method of determining the role of some of these intermediate products is to observe the effect of adding certain supposed intermediate or precursor, substances, for example acetaldehyde, on the amount of derivatives of acetaldehyde produced. As a result of such studies we know most or many of the end-products resulting from glucose metabolism by certain species, for example *Aerobacter aerogenes,* so that we may plot out a sequence of chemical steps (see Fig. 134).

In the case of *Aerobacter aerogenes* the following final products of glucose dissimilation have been demonstrated to occur: hydrogen, carbon dioxide, ethyl alcohol, acetic acid, acetyl-methyl-carbinol, 2.3-butylene glycol, trimethylene glycol, lactic acid, glycerol and succinic acid. *Acetaldehyde, pyruvic acid* and *acetyl-methyl-carbinol* have been demonstrated as intermediate, transitory substances. Whether the reactions that occur in their formation are exactly as indicated in figure 134 is not certain, but some seem probable.

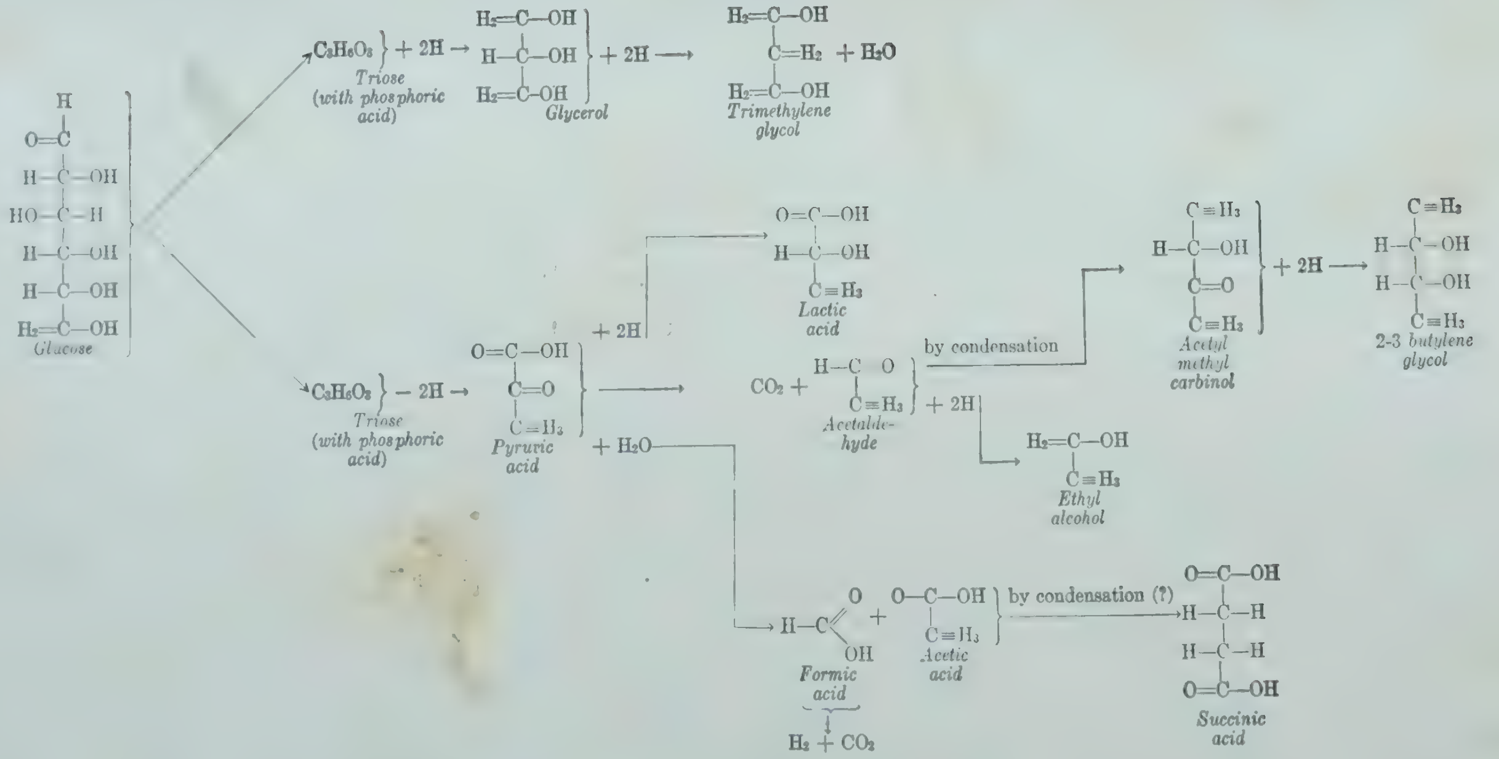

Glucose
Triose (with phosphoric acid)
+ 2H
Glycerol
+ 2H
Trimethylene glycol
+ H2O
Triose (with phosphoric acid)
− 2H
Pyruvic acid
+ 2H
Lactic acid
CO2 +
Acetaldehyde
by condensation
Acetyl methyl carbinol
+ 2H
2-3 butylene glycol
+ 2H
Ethyl alcohol
+ H2O
Formic acid
H2 + CO2
Acetic acid
by condensation (?)
Succinic acid

Fig. 134. Diagram of the dissimilation of glucose.

As shown above, some of the end-products of glucose metabolism are acids and alcohols, which may eventually kill the bacteria in the culture. Some of the acids may under certain conditions of aeration be broken down further so that after a time the reaction of the culture reverts to alkalinity partly through the formation of carbonates.

Some of the products of bacterial metabolism are valuable in industry, ethyl alcohol and butyric acid for instance.

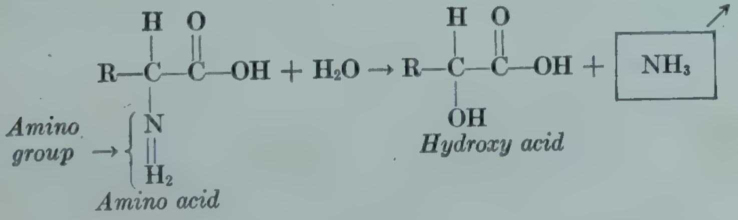
Aerobacter has its own distinctive end-products by which it may be distinguished from closely related species. One of these end-products is acetyl-methyl-carbinol which is easily tested for in a culture tube. Other organisms may often be identified by analogous tests for other metabolic byproducts.

The scheme shown in figure 134 is a mere diagram, not in any sense an equation. Many of the intermediate steps are not shown and are not known. The diagram shows mainly end points and products known to result from the dissimilation of glucose, and their possible precursors or substances from which they are derived.

The utilization of glucose as an energy source may be thought of as involving, first, the degradation to lactic acid or pyruvic acid. This does not require free oxygen. Second, the breakdown of pyruvic acid to simpler substances somewhat as outlined below. Nearly all of the energy available in the glucose is liberated in the latter series of reactions. As the oxidation of pyruvic acid proceeds inorganic phosphate is combined as organic phosphate and much energy is "built into" the organic phosphate bonds. The energy taken up in the phosphate bonds is used in the cell for mechanical work, as motility, osmotic work, synthesis, etc., when the phosphate bonds are later broken. As will be seen later, amino acids and fats are disposed of in the cell in a somewhat similar manner.

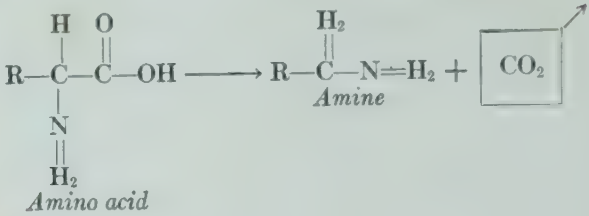
Amino Acid Dissimilation. Various schemata have been drawn, analogous to that for glucose decomposition by *Aerobacter,* suggesting how the amino acids may be broken down and utilized.[18, 19] Here again, the end-products give some clue to the nature of the processes. Various intermediate products of the reactions involved have been isolated or demonstrated, and others plausibly postulated. Four simplified types of reaction may be listed as follows:

(*a*) Hydrolysis, resulting in deamination with liberation of ammonia:



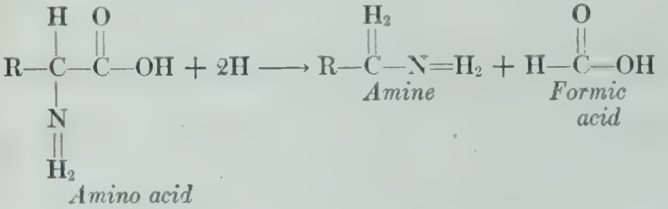
$$R-\underset{\underset{NH_2}{|}}{\overset{\overset{H}{|}}{C}}-\overset{\overset{O}{\|}}{C}-OH + H_2O \rightarrow R-\underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{C}}-\overset{\overset{O}{\|}}{C}-OH + \boxed{NH_3}\nearrow$$

(*b*) Decarboxylation (a molecular rearrangement):



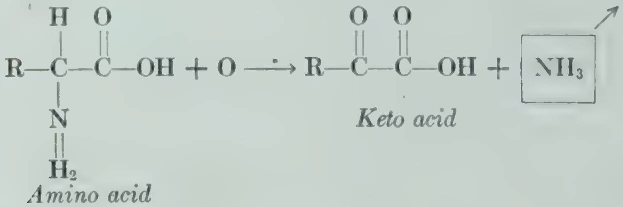
$$\mathrm{R{-}\underset{\underset{\underset{H_2}{\|}}{N}}{\overset{H}{C}}{-}\overset{O}{\overset{\|}{C}}{-}OH \longrightarrow R{-}\overset{H_2}{\overset{\|}{C}}{-}N{=}H_2 + \boxed{CO_2}\nearrow}$$

(*c*) Hydrogenation (reduction):



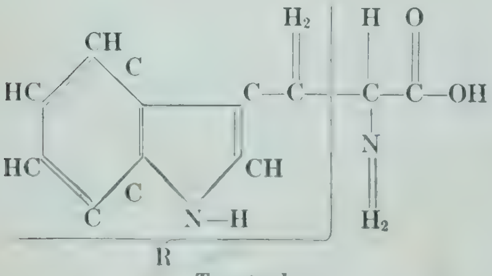
$$\mathrm{R{-}\underset{\underset{\underset{H_2}{\|}}{N}}{\overset{H}{C}}{-}\overset{O}{\overset{\|}{C}}{-}OH + 2H \longrightarrow R{-}\overset{H_2}{\overset{\|}{C}}{-}N{=}H_2 + H{-}\overset{O}{\overset{\|}{C}}{-}OH}$$

(*d*) Oxidation (with deamination or liberation of ammonia):

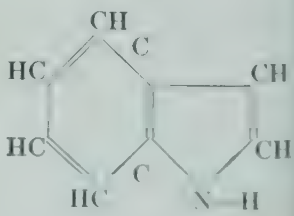


$$\mathrm{R{-}\underset{\underset{\underset{H_2}{\|}}{N}}{\overset{H}{C}}{-}\overset{O}{\overset{\|}{C}}{-}OH + O \longrightarrow R{-}\overset{O}{\overset{\|}{C}}{-}\overset{O}{\overset{\|}{C}}{-}OH + \boxed{NH_3}\nearrow}$$

In these formulae R in the amino acid may be no more than a CH_3 group, or it may be a very complex radicle like that in tryptophane:



Tryptophane



*Indol**

The oxidative deamination of tryptophane results in the formation of indol acetic acid which is a potent growth hormone for many green plants, favoring root growth especially.

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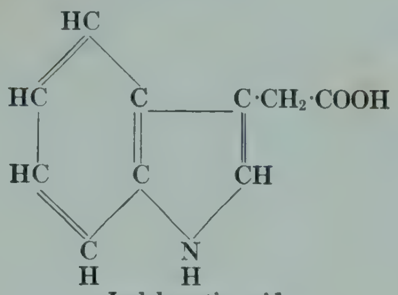
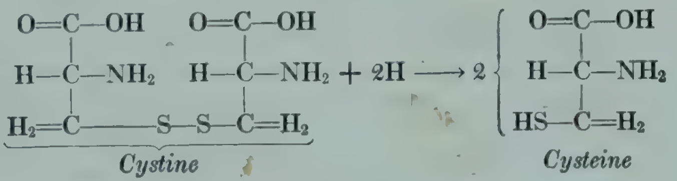
* The molecule of indol is placed beside that of tryptophane for comparative purposes. Some important bacteria are identified by the fact that they can split off the indol group from the tryptophane molecule.

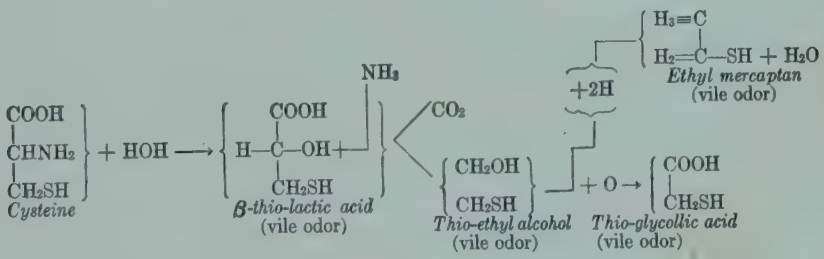


Indol acetic acid

Note that, in two of the reactions given above, (*a*) and (*d*), ammonia is split off. This is a very common occurrence in protein decomposition, and helps to contribute to the odoriferousness of the process and also to the fertility of the soil containing it (see sections on *Nitrosomonas* and nitrogen cycle, pages 601 and 609). The keto and hydroxy acids also contribute to the odors of protein decomposition. Other foul odors commonly sensed in putrefaction of protein are due to volatile sulfur compounds—the mercaptans, skatol, hydrogen sulfide and others.

These may be derived from sulfur-containing amino acids, like cystine, as follows:

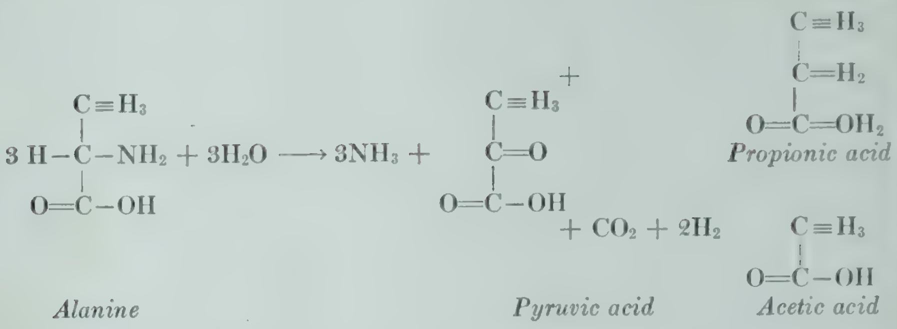

Cystine
Cysteine


Cysteine
β-thio-lactic acid
(vile odor)
Thio-ethyl alcohol
(vile odor)
Thio-glycollic acid
(vile odor)
Ethyl mercaptan
(vile odor)

The reactions in this schema are partly hypothetical but the end-products have been demonstrated.

Some of the amines (reaction *b*) produced as a result of protein decomposition were formerly called *ptomaines,* believed to be poisonous if eaten. This is very doubtful.

A schema showing one way in which an amino acid such as alanine may be decomposed and used as a source of carbon and energy is of interest because the products are much the same as those resulting from glucose metabolism by some bacteria.



$$3\begin{array}{c} C{\equiv}H_3 \\ | \\ H-C-NH_2 \\ | \\ O{=}C-OH \end{array} + 3H_2O \longrightarrow 3NH_3 + \begin{array}{c} C{\equiv}H_3 \\ | \\ C{=}O \\ | \\ O{=}C-OH \end{array} + \begin{array}{c} C{\equiv}H_3 \\ | \\ C{=}H_2 \\ | \\ O{=}C{=}OH_2 \end{array} + CO_2 + 2H_2 \quad \begin{array}{c} C{\equiv}H_3 \\ | \\ O{=}C-OH \end{array}$$

Alanine Pyruvic acid Propionic acid Acetic acid

Hydroxy acids like lactic acid, and similar compounds, undergo similar changes.

Not all bacteria decompose the same amino acids, nor do they attack amino acids in the same manner. As in carbohydrate decomposition, tests for certain end-products like indol (see above) are used in the identification of certain species of bacteria.

That these pathways of chemical transformation are not fixed and inviolate is shown by the work of Gunsalus and Campbell who entirely altered the nature of the end-products of fermentation of *Streptococcus faecalis* by furnishing more highly oxidized substrates than glucose, namely, citric acid.[20]

Aerobic and Anaerobic Metabolism. Since oxidations serve as a source of energy, it might be supposed that all bacteria require free oxygen. Many species, like *Pseudomonas fluorescens* and *Bacillus subtilis,* are of this type. They cannot get along without free access to air. These are spoken of as *strict aerobes.* However, other species which utilize atmospheric oxygen can also grow quite well without free oxygen. These are called *facultative anaerobes.* They use combined oxygen for cell synthesis, and do not need it in respiration. Many common species, like *Escherichia coli, Streptococcus lactis* and *Staphylococcus aureus,* are facultative in respect to oxygen. These make use of respiratory mechanisms like dehydrogenation, molecular rearrangements, etc., which do not involve atmospheric oxygen. Still other species, such as *Clostridium tetani, Actinomyces pseudonecrophorus,* and *Bacteroides* sp., not only are able to grow without free oxygen, but absolutely require its entire absence and are very adversely affected by its presence. Such species are called *strict* or *obligate anaerobes.* Pasteur was first to discover this phenomenon. He called it anaerobiosis ("life without air"). Special apparatus, some forms of which are discussed farther on, is necessary for studies of strict anaerobes in the laboratory. It may be noted that heterotrophic, autotrophic and intermediate metabolic groups may contain aerobes, anaerobes, or species which are facultative.

Some bacteria seem to be adapted to oxygen tensions somewhat less than that of the atmosphere, yet greater than that of complete or almost complete absence of oxygen. Such organisms are said to be *microaerophilic.*

Anaerobic respiration is much less efficient than aerobic respiration. For example, yeast may completely oxidize sugar in the presence of air to H_2O and CO_2. This releases 673,000 calories. Under anaerobic conditions the sugar is changed to alcohol and CO_2. This releases only 26,000 calories. Much of the energy of the sugar remains in the alcohol. The anaerobic process yields enough energy to support anaerobic life if a *large amount* of sugar is present. Obviously anaerobic life requires larger amounts of energy sources than aerobic to get the same amount of energy.

Anaerobes, Hydrogen Peroxide and Catalase. The sensitivity of strict anaerobes to free oxygen has long puzzled bacteriologists but may be explained on various bases, although the exact and complete truth of the matter is not yet available and probably differs for different species. One explanation is as follows: many bacteria give off hydrogen peroxide as a metabolic by-product when cultivated in the presence of free oxygen. As is well known, hydrogen peroxide is unfavorable to many bacteria and is often used as a disinfectant. Most bacteria, then, would soon succumb to their own deleterious by-product had they not some mechanism for protecting themselves from it. Many bacteria able to grow in the presence of air actually do protect themselves from hydrogen peroxide by the production of the enzyme *catalase* which decomposes hydrogen peroxide as fast as it is formed. The strict anaerobes, on the contrary, produce no catalase, hence they are killed by their own metabolic product when cultivation is attempted in situations where there is more than a certain, minimum amount of free oxygen. There are some aerobic bacteria which form hydrogen peroxide yet which do not produce catalase. But it is found that these species are relatively insensitive to hydrogen peroxide.

Some of the strictest anaerobes may be cultivated when freely exposed to air provided the medium contains some substance which possesses a *strongly reducing,* or oxygen-combining, power which takes care of the excess oxygen so that peroxides are not formed in bactericidal amounts.

The ability of several organisms, including one strict anaerobe (*Cl. novyi*), to produce H_2O_2, can be shown by using the method described by McClung, Billman and Reed.[21]

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Chapter 16

IMMUNOLOGY AND MICROBIOLOGY

I. Principles and Methods

In a previous chapter we have spoken of defensive mechanisms of the living body against pathogenic microorganisms. The study of these defensive mechanisms is termed Immunology. This science is a broad one involving every sort of physical and chemical phenomenon occurring in living systems. The methods of immunology are so widely used as laboratory tools and are based on such fundamental biological principles that one must have a clear grasp of them before a detailed study of microbiology will prove profitable.[1, 2]

Blood. Before discussing the nature of immunity and the methods of studying it, it will be advisable to devote a little space to a description of blood and its constituents, since these are much used in immunological investigations.

The blood is conveyed by the circulatory system to the remotest vascular parts of the body where it carries food to the tissue cells and removes their waste products. Blood may carry death and destruction to certain body cells if toxin-producing bacteria gain a foothold somewhere in the body and secrete their poison (toxin) into the tissues, where it is absorbed by the blood and lymph (see page 309). On the other hand, blood carries with it at all times substances and special cells which help to combat bacteria and toxins. The study of these protective substances and cells is part of the science of *immunology* and a knowledge of them is important to an understanding of host-parasite relationships.

Important Blood Constituents. For the purposes of this discussion blood contains four important constituent parts. First, there is the *plasma,* the yellowish, transparent, fluid part of blood, consisting of a solution of proteins, salts and other soluble substances as well as food for, and wastes from, the body cells. The plasma has in solution, also, the substances necessary to the formation of a *clot* or *fibrin.*

Fibrin Components. When freshly drawn blood is allowed to stand in a test tube or flask, it will be found, after a few minutes, to have solidified or clotted. The material responsible for this forms a fine fibrous *network* in the blood after the blood leaves the body. The meshwork is composed of a substance called *fibrin*, a highly complex protein which is very elastic. The fibrin clot soon shrinks to about half the original volume of the blood, squeezing out of its meshes the fluid portion of the blood, much as a wet, contractile sponge would exude water. Most of the blood cells are caught and held in the fibrin (Fig. 135). Associated with fibrin production are small, stellate, non-cellular bodies called *platelets*. Their nature is not yet fully understood (Fig. 136 *F*).

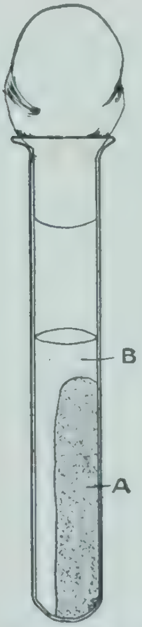


Fig. 135. Tube with clot (*A*) which has shrunken, enmeshing the blood corpuscles and exuding clear serum (*B*).

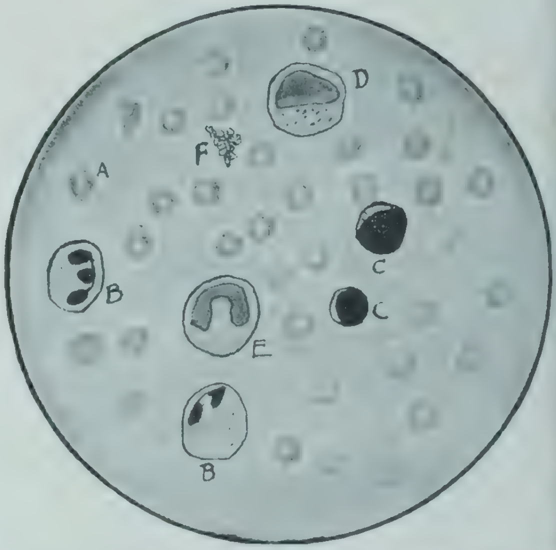


Fig. 136. Stained smear of normal human blood showing different kinds of corpuscles. *A*, Red corpuscles; *B*, white corpuscles (polymorphonucleated leukocytes); *C*, small lymphocytes (another kind of leukocytes); *D* and *E*, other kinds of leukocytes (large lymphocytes); *F*, blood platelets. × 900.

Serum. The fluid portion of blood, after it is separated from the fibrin, is called *serum*. Since fibrin usually enmeshes the blood corpuscles, serum is yellowish and transparent, like plasma.

Erythrocytes. In the plasma, before clotting, are suspended the red corpuscles (or *erythrocytes*), non-nucleated cells (when mature) which give to blood its opacity and red color and which carry oxygen from the lungs to the tissues, and carbon dioxide from the tissues to the lungs. The color of erythrocytes is due to the red substance, *hemoglobin*, which they contain. When they are ruptured by plasmolysis or other means, the hemoglobin is released and the plasma becomes transparent and red. The cells are said to have *hemolyzed.* Certain bacteria (*e.g.,* beta-type hemolytic streptococci and *Bacillus subtilis*) are very active in producing hemolysis.

Leukocytes. In addition to erythrocytes blood contains colorless, ameba-like cells called *leukocytes* ("white cells") or *phagocytes* (*phago* = eat; *cyte* = cell). These are relatively large cells which have a definite nucleus and means of locomotion like amebae. They act as scavengers and "policemen" in the blood (Fig. 136 *B, C, D, E*). Like amebae, leukocytes can ingest food particles, and in the body they serve by ingesting and destroying any small foreign particles, such as bacteria, dead body cells and the like, that may gain entrance to the tissues or blood stream. The leukocytes can leave the blood vessels and congregate in the tissues wherever any irritation exists. There are other cells having the power to ingest and destroy bacteria, etc., in the blood. These other cells are parts of tissues and remain in place in liver, spleen, blood vessels, etc.

Phagocytosis. Centers of inflammation such as the bacteria in a boil, or a splinter of wood or other foreign particle, exert a strong attraction for leukocytes, which concentrate in such areas. The nature of this attraction is not fully known. It is called *chemotaxis*. The white cells try to dispose of the foreign particles by ingesting them and also by giving off enzymes which will kill and destroy or digest them. Often many leukocytes are in turn killed by poisons of the bacteria they ingest. Thus, the white, creamy material in a boil or other infected lesion or around a "festering" splinter is made up largely of dead and living white corpuscles, dead and living bacteria, tissue debris, lymph, fibrin and serum. It is called *pus* and the dead leukocytes in it are called *pus* cells.

Lymph. Lymph is very much like blood which has been deprived of its corpuscles by passing through the thin walls of the smaller blood vessels as through a fine filter. The fluid thus seeping out of the vessels travels slowly in the fine spaces surrounding the blood vessels and other organs and eventually collects from all parts of the body in a large drainage vessel and is returned to the blood stream.

Foreign Substances. As mentioned above, the phenomena of immunity depend on the reaction of the animal body to foreign substances which gain entrance to it. For the purposes of this discussion, the interior of the gastro-intestinal tract is considered *external* to the body proper, as it is open to the outer world at both ends and has no direct connection with the principal body cavities. We shall speak, therefore, only of foreign substances which get into the body *parenterally* (*i.e.,* other than through the gastro-intestinal tract). This includes, generally, substances or bodies injected into the tissues with hypodermic needles, or introduced by means of splinters or wounds, or forced into the peritoneal or pleural cavity, or released or absorbed from mucous and other surfaces into the blood, or in any situation where they are not exposed to the digestive enzymes of the mouth, stomach or intestines.

Reaction to Foreign Substances. When foreign substances are solid, inert and insoluble particles like wooden splinters, shrapnel fragments, sand or glass, the tissues respond locally with inflammation or pus formation to expel them, or forms walls of fibrous connective tissue to incarcerate them, but there is no demonstrable permanent general change in the chemical or physical composition of the blood or tissues. When the foreign substances happen to be *soluble,* complex organic substances, mainly of the class of proteins, or irritating microorganisms, such as typhoid bacilli, the tissues respond not only locally with inflammation and other changes, but the whole body may be aroused and the tissues everywhere, and the blood, undergo a marked change in chemical or physical constitution, or both, of such a nature as to repel or destroy the foreign protein complexes or the irritating microorganisms and their poisonous metabolic products (toxins) if any. The reaction is often so violent as to cause fever. The infective agents tend to die out, and the patient recovers but is a "changed man."

Mechanisms of Protection. Before proceeding further into a detailed discussion of these changes we may briefly survey the various types of mechanism by which the animal host is protected from injury by foreign substances, microorganisms, etc. These may be listed as follows:

1. *Genetic.* Racial or species resistance, illustrated by the facts that horses do not have measles, man does not contract fowl pox, certain races of white mice are highly susceptible to yellow fever while others are not, and so on.
2. *Physiological* (*i.e.,* dependent on the physiological state of the individual and the reactions of his body cells).
   a. *State of health.* Persons weakened by long overwork, starvation, exposure, age, disease, etc., are seemingly more susceptible to various infections such as tuberculosis, pneumonia, etc.
   b. *Localizing or binding action of tissue cells.* This is a little-understood property manifested by the ability of tissues (*e.g.,* subcutaneous tissues, etc.) to hold microorganisms and their toxins so as to prevent their dissemination throughout the body.
   c. *Phagocytosis.* This has been described briefly above, in connection with leukocytes and fixed tissue cells. Phagocytic cells, whether free or fixed, take up all sorts of foreign matter,

as blood-pigments, dead blood and tissue cells, microorganisms, etc.

With respect to leukocytes, certain soluble substances, often called *opsonins,** in the blood seem to aid them greatly in taking up microorganisms. Opsonins appear to affect the microorganisms in such a manner that they are much more readily ingested by the leukocytes. Opsonins appear to be *specific antibodies* (see paragraphs below) which act in conjunction with an enzyme-like substance of the blood similar to, or identical with *complement* (see page 319). The mode of action is not clear.

d. *Production of antibodies.* After recovery from certain diseases, such as diphtheria, whooping cough, typhoid fever, etc., the patient is so changed that he may never again, under *ordinary* circumstances, acquire the disease. He is said to be *immune* to that disease. Upon proper tests, his blood is found to contain substances called *antibodies,* produced by certain body cells, which neutralize the toxins or unfavorably affect the bacteria, or both, and so protect him from the disease, and his tissues are found to be relatively resistant to the harmful effects of those organisms or their toxins.

In some cases, as under experimental conditions, foreign organic substances of a harmless nature may gain entrance to the tissues by injection. If these are soluble proteins, for example, egg white, or animal serum, the body reacts in much the same way as toward bacterial toxins or the proteins of infecting organisms, *i.e.,* durable changes occur in the tissues which are directed toward the destruction and removal of the foreign substance in question. Such reactions to harmless substances are included, for convenience, in the general term immunity. While much of the discussion which follows will deal with infectious microorganisms, because much of our knowledge of the subject of immunity is derived from the researches of microbiologists interested in immunity to disease, the principles involved must be thought of in a broad biological sense as applying generally to foreign substances (usually proteins) of diverse origins.

Often antibodies continue to be produced by the body cells long after the stimulus which excited their production disappears, and it may be to this that prolonged immunity, such as follows mumps, measles and diphtheria, is due. However, it must also be emphasized that immunity often seems to be dependent on certain properties of the tissue cells themselves, which are not related to the presence of demonstrable antibodies in the blood stream. Conversely, the presence of certain antibodies easily demonstrable in the blood does not imply

* Opsonin is from a Greek root meaning "to prepare food."

or guarantee immunity. It is important to bear these facts in mind. The great value of *tissue immunity* and *phagocytosis* as defensive mechanisms is generally overshadowed by the more easily studied serum or humoral immunity, *i.e.,* antibodies.[3]

Specificity of Immunity. Immunity is said to be *specific.* That is, a person who recovers from diphtheria is immune to diphtheria. He is not, however, necessarily immune to other diseases, such as typhoid fever or whooping cough. In the case of nonbacterial proteins, the principle of specificity holds with equal regularity. Egg-white antibodies will not react with hookworm proteins, or horse-serum antibodies with rabbit serum.

This specificity is dependent on the chemical and physical structure of the antibodies involved, a matter which will be discussed farther on. It is closely related to and dependent upon the chemical and physical structure of the foreign substance stimulating the formation of antibodies.

Antigens. Almost any protein, and a few carbohydrates, soluble in water, will stimulate the production of antibodies or immunity against themselves when injected into any animal species other than that from which they came. Such proteins or carbohydrates are spoken of as *antigens* (*anti* = against; *gen* = produces). Infectious microorganisms also act as antigens.

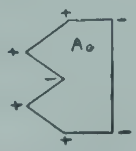
Antigens, like antibodies, are specific. That is, each antigen engenders only one sort of antibody, and will react *only* with that antigen. We therefore say that *antigen-antibody reactions are highly specific.* The antigen-antibody reaction, accordingly, gives us a method of high precision in the study of proteins and certain protein-complexes. Several methods of using this instrument in microbiology will be discussed after we give some attention to the nature of specificity.

Nature of Specificity. It is very simple to state that antigens and antibodies are *specific* for each other, but the explanation is far from simple. However, several pertinent facts may be of help in forming a concept of the relationships.[4, 5, 6, 7, 8] First, as mentioned above, specificity is a matter largely of chemical and physical structure. For example, a synthetic "protein" antigen (of which several have been prepared experimentally by combining protein with certain organic radicles) having a given chemical structure (*e.g.,* $NH_2C_6H_4 \cdot AsO_3H_2$—Protein) will, upon injection into the body, engender antibodies in the serum which react *only* with that compound. But if by chemical treatment we alter the antigen (say with an SO_3H group in place of the AsO_3H_2 group), no reaction with the original antibody occurs. Almost any sort of chemical alteration in a protein will alter its specificity. A slight reaction may occur if, instead of substituting an SO_3H group, we introduce, say, a Cl atom in place of the NH_2 group. Then the antibodies produced in response to the original antigen are said to *cross-react* with the chlorinated antigen. Many cross-reactions occur in nature between closely related antigens. *Hydrolysis* or *destruction of solubility* of an antigen are likely to result in complete loss of antigenic properties.

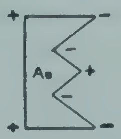
As a corollary of specificity resulting from chemical and colloidal structure of antigen and antibody, we may postulate certain definite distributions of positive and negative electrical fields of force on the surface of the colloidal particles or molecules which constitute antigens, due to electron arrangements upon the constituent atoms. Any change in chemical constitution of the antigen, such as introduction of a Cl atom or SO_3H group, will affect the distribution or relative intensities of these electrical fields or charges on the antigen molecule surface.

Now an antibody molecule is also a complex, colloidal, protein molecule and we may imagine its surface to have a pattern of electrical fields, positive or negative, analogous to those of the antigen, but *arranged as a mirror image* of those of the antigen. These electrical charges on the molecules are of the utmost importance. Not only do they determine the "energy-pattern" of the molecules, but the colloidal particles of either antibody or antigen are maintained in suspension-solution partly by the unneutralized portion of their electrical charge and partly by their small (colloidal) size and their lyophilic nature, *i.e.,* diffuse boundary layer.

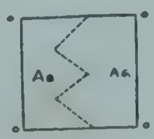
When mixed in a test tube, the antigen and antibody colloids coming into contact could, according to this hypothesis, if specific for each other, orient themselves with respect to their positive and negative charges so that an absolute "fit" is obtained. Thus, we may imagine an antigen (*Ag*) to be represented by the figure:



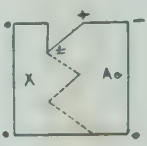
its corresponding antibody (*Ab*) by the figure:



and the antigen-antibody combination by the figure:



The compound colloidal particle forming upon reaction of these two would be an *electrically neutral* and *very large* one and, as a result, would become unstable in solution (a lyophobe colloid) and would therefore go out of solution. In a test tube reaction, it would become *visible* as a cloudy *precipitate* (Fig. 137). We would have what is known as a "*precipitin reaction*." A weak cross-precipitation might occur between antigen *Ag* and some antibody (*X*) closely similar to *Ab*, but not exactly like it:



This specific physicochemical interrelationship between an antigen and its antibody is commonly likened to the relationship between a lock and key. Some locks are absolutely specific for their own keys. This may be altered by changing the structures of such locks. They may then open to several other keys of somewhat similar structure. It will be noted that we have to depend on analogies and speculation to some extent to explain the observed facts.

A few additional points concerning antigen-antibody reactions are worthy of mention at this point. First, the union of the two is firm but not always irreversible since they may be separated by certain procedures.

Second, *surface structures are in great part the determining forces.* It is the arrangement of the *surface* molecules, and charges of the *surface* colloids which dominate reactivity and specificity. This is of *especial importance* in relation to cells such as bacteria. The *surfaces* of the cells are of greatest significance in reacting with antibodies, since antibodies, being colloids, cannot gain access to the interior of the intact cell. Change in chemical or physical structure of the *surface*, for example, the presence of a capsule, determines alteration of specificity of the antigen.

Third, these reactions proceed in two stages: (*a*) *combination*, presumably due to *adsorption*, especially on cell surfaces. This may proceed rapidly at temperatures around 37° C.; (*b*) *visible precipitation*, cell *lysis* or other effect. This often occurs slowly and may often be demonstrated best after twelve to eighteen hours at 4 to 6° C. The presence of electrolytes in the solution is often necssary for the second stage (Fig. 137).

Complexity of Natural Antigens. By means of synthetic chemistry and the precipitin reaction, it has been possible to delve deep into the physicochemical relationships of antigens and antibodies and the reactions of immunity. This has involved the study of very pure substances. In nature, antigens seldom occur as pure compounds separate from others and this greatly increases the difficulties surrounding the study of antigen-antibody reactions involving such antigen mixtures as bacterial cells. A cell may consist of many antigens (*e.g.*, cytoplasm, flagella, capsule, etc.). The serum of a person or animal, following injection with such cells, contains a mixture of antibodies active against each separate antigen.

To complicate matters further, the cells of two different, but related, species may contain certain antigens in common so that antisera will cross-react with both kinds of cell.

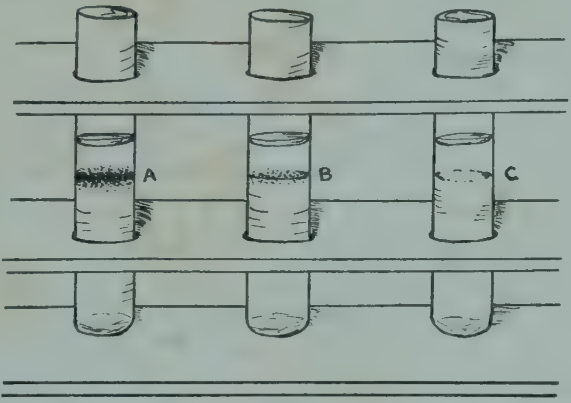


Fig. 137. Precipitin reaction between an anti-cow-protein serum and its antigen. The precipitin-containing serum is in the lower part of the tubes and the antigen has been placed above it in such a manner that the two are in contact but do not mix extensively. A strong reaction is seen at *A*. At *B*, the cow-protein antigen has been replaced by goat antigen and a very weak cross-reaction is seen. At C the antigen has been replaced by saline solution and no precipitate is seen at the junction of the two fluids. This is a "control" tube.

For example, three related species of dysentery bacilli may each contain four antigens as follows:

Species I	A/B/C/D
Species II	C/D/E/F
Species III	E/F/G/H

Obviously, upon injecting species I into a rabbit, antibodies a, b, c, d, will be engendered, corresponding to antigens A, B, C, D. Upon injecting species II into another rabbit, antibodies c, d, e, f, will be called forth. Likewise, species III will stimulate production of antibodies e, f, g, h. Now, the serum of rabbit I will react best of all with bacterial species I when these bacteria and serum of rabbit I are brought into contact. Serum II will similarly react best with species II, and serum III with species III. However, since serum I contains antibodies c and d, it will cross-react to some extent with bacterial species II, since the latter has these antigenic compounds in common with species I. There will be no cross-reaction between serum I and antigen III, but serum II will cross-react with species III. Such cross-reactions are commonplace and are the basis of possible methods of producing immunity to disease by injections of non-specific antigens.[9 15]

Even such unrelated organisms as *Brucella* (the cause of undulant fever) and *V. comma* (the cause of Asiatic cholera) may contain antigens common to both.[15]

Antibody Adsorption. If a given volume of serum I (say 5 ml.) be mixed with a heavy suspension of cells of bacterial species II, then antibodies c and d, and antigens C and D will combine, leaving antibodies a and b still free in the serum. By means of centrifuging, the bacteria with their attached antibodies c and d can be removed, leaving serum I free from antibodies c and d and *specific* with regard to species I; *i.e.*, it will no longer cross-react with species II and will react only with bacteria containing antigens A and B. If we further adsorb antibody b by treatment of the serum with some species having only antigen B in common with species I, then we obtain a pure A serum. Many such pure and specific sera are thus prepared and it has been possible to make very extensive analyses of the antigenic structure not only of bacteria, but of antigens from higher plants and animals, and to detect antigenic relationships hitherto unsuspected.

Antigenic Structure of Bacterial Cells. Relatively little is known of the localization or nature of the different antigenic components of most living cells. With respect to bacteria, we know that certain antigens are quite widely distributed in certain groups. Thus, certain of the cell-body or somatic antigens (so-called O antigens—see page 173) of many of the typhoid-paratyphoid organisms are shared in common. The antigens in the flagella (the so-called H antigens—see page 173) are not widely shared, but are specific to the type or species.*

In those species which possess capsules, the capsular substance dominates the surface and often determines antigenic specificity of a very high order. But antigens of capsules may also be shared with wholly dissimilar species.

The localization of antigenic specificity in the capsular substance of such organisms as influenza bacilli, streptococci and Friedländer's bacillus is a striking phenomenon. Stripped of this specific surface antigen, the exposed proteins of the naked cells cross-react very widely with antisera of related and often unrelated species. The somatic or cellular antigens of all pneumococci are immunologically alike. Antibodies for one react equally well for all. But the antibodies of the capsules are not all alike and it has been found that there are at least thirty-two different kinds of pneumococcus capsular antigens. Each determines a different type of pneumococcus. This is part of a general biological phenomenon.

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* Except in certain phases of variation called the "group phase" when they become more like O antigens.

Specificity or other immunologic behavior of an *intact* microorganism in the presence of antibodies may be thought of as depending upon whatever antigenic component of the cell dominates the surface at the time of the reaction. However, the antibodies engendered in the serum of an animal are often determined apparently by both surface and deep antigens. It may be imagined that once in the body the antigenic bacterial cell breaks down so that all of its various proteins exert their antigenic effect, whereas only the antigens of the surface of *intact* cells come into contact with antibodies.

Types of Antibody Response and Reaction. There are different manifestations of antibody action, some of them demonstrable in test tubes as visible reactions, some of them not visible except indirectly by secondary tests for their presence such as complement fixation or animal protection tests. The different manifestations of antibody action are spoken of as though different antibodies were involved, but there may be fewer antibodies than types of reaction. The kind of antibody action manifested seems to depend in great part on the kind and size of antigen molecule or cell, the physical conditions of the suspending fluids, electrolytes, and other factors. For convenience, we shall speak of "types of antibody," including in that term "types of antigen-antibody reactions." Among the types of antibody are antitoxins, cytolysins, agglutinins and precipitins.

Antitoxins. When bacteria gain a foothold in the body and secrete toxin into the blood, the toxin, being protein-like, stimulates the production of an antibody. This antibody neutralizes the toxin and is therefore called *antitoxin*. The reaction may be thought of as resulting in precipitation in which the particles of precipitate, as a rule, are too small to be seen. Under certain conditions in which the quantitative relations are carefully adjusted visible minute flakes or floccules are produced (see below). The reaction is quantitative but seems to obey primarily the laws which govern adsorption rather than chemical combination.

Quantitative Relations. As mentioned above, when exactly the right proportions of toxin and antitoxin are brought together in a test tube a visible precipitate or flocculation occurs. This fact is made use of in determining the concentrations or "strengths" of toxin or antitoxin. For example, we may set up a row of ten tubes. In the first we put serum containing a quantity of antitoxin arbitrarily spoken of as 2 units.* In the next we place 4 units, in the next 6 units, and so on. We then add to each tube a fixed amount, say 1 ml. of filtered broth culture which contains toxin in unknown amount. After a short time flocculation appears in one of the tubes, let us say the sixth tube. Since this contained 12 units of antitoxin, we have a measure of the potency of the toxin broth. We say that it contains 12 flocculation units (12Lf) of toxin per ml. This is an arbitrary unit of potency.

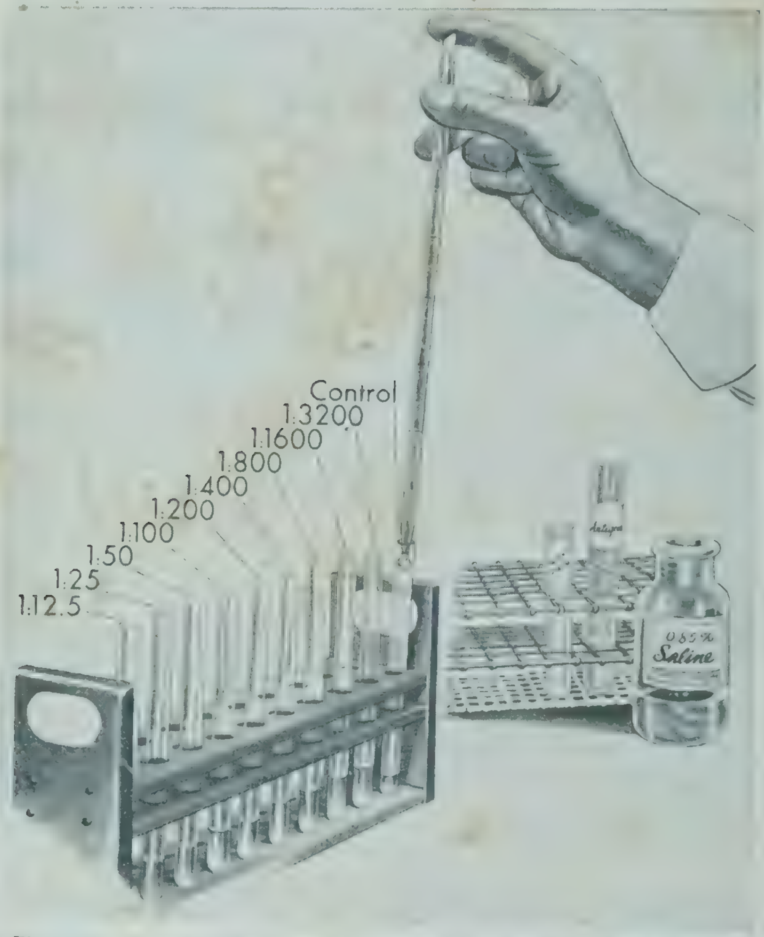

Fig. 138. Technic of test tube flocculation and other serological tests. (Seminar, vol. 6.)

Zone Reaction. The above reaction illustrates a curious manifestation called a zone phenomenon (sometimes called pre-zone and post-zone, or *zones of inhibition*).

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* A unit (*approximately*) of diphtheria antitoxin is the least amount necessary to protect standardized (250 to 300 gm.) guinea pigs against 100 minimal lethal doses (M.L.D.) of diphtheria toxin. An M.L.D. kills about 75 per cent of such pigs in from four to five days.

This occurs not infrequently in immunological tests. It depends on the fact that, for maximal amount of visible precipitate to be formed most rapidly optimal amounts of antigen and antibody must be present. If there is too great an excess of either, less precipitate or none at all will appear, although a certain amount of antigen-antibody combination may occur without *visible* precipitate, or with the formation of soluble complexes. Many factors are involved, among which are type of animal serum used, nature of antigen and antibody, *p*H, electrolytes, and so on.

The practical importance of such zone phenomena is that, in determining the precipitin activity of an antibody-containing serum or an antigen by testing it in a series of dilutions (see Fig. 138) the series should be made sufficiently long, and the intervals between dilution steps not too great, or the zone of optimal reaction may not be reached or may be passed over and no reaction seen. The same considerations hold for many other serological tests involving antigen-antibody reaction. They are of especial importance in the serological diagnosis of syphilis.

Cytolysins and Complement. The proteins of which a given bacterial cell is composed may be different from the exotoxin formed by that cell and may stimulate the body to produce antibodies which assist in the dissolution (or *lysis*) of the bacterial cell itself. These antibodies are termed *cytolysins* (*cyto* = cell; *lysin* = dissolver). They are also called, because of the way in which they combine with the antigen and the serum enzymes, *sensitizers.** Their action is a little more complex than the simple neutralization of toxin by antitoxin and may be described briefly as follows:

The cytolytic antibody first combines with the foreign cell that called it into being. The cell may be a bacterium, an erythrocyte or a cell of other nature. This simple combination is, however, not sufficient to destroy the cell, and there is no visible reaction. A second substance called *complement,* which is *normally* present in all mammalian blood and which is entirely *nonspecific,* is necessary to *complete* (hence *complement*) the lytic action. It, too, combines with the cell which, in order for it to act, must already have been *sensitized* by the cytolytic antibody. *Lysis,* or cell dissolution, then results.

Complement cannot, *by itself,* destroy foreign cells, but must act through the intermediation of the sensitizer. The sensitizer is a specific antibody, but *complement is nonspecific;* it helps *any* sensitizer to complete its work. After complement has combined with the sensitized cell it is no longer active and is said to be *"fixed."* It is adsorbed on the sensitized cells.

The Complement-fixation Reaction. This inactivation, or fixation of complement was first demonstrated in 1901 by Bordet, a famous Belgian scientist. Bordet discovered that if the serum of a person who had recovered from bubonic plague (and therefore containing anti-plague cytolysin) were mixed with plague bacilli, the free complement in the serum was all used up or fixed in destroying the bacilli. When the complement was tested for, by an appropriate method, none was to be found free in the serum.

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* The term amboceptor is sometimes used for sensitizer.

Complement fixation may occur in reactions which do not necessarily result in cell lysis. Complement is readily adsorbed on the surfaces of any finely divided particles, visible or invisible. For example, complement is adsorbed by the floccules resulting from the interaction of toxin and antitoxin described above (see page 317). Complement may be fixed *in any antigen-antibody reaction,* such as precipitin, or toxin-antitoxin reactions but is not a necessary component of such reactions as it is in cytolysis.

This phenomenon of complement fixation is used today by immunologists and microbiologists to diagnose disease by the identification of certain antigens causing disease, or to detect and identify their corresponding antibodies which appear in the blood as a result of disease.

For example, if antibody, antigen and complement be mixed in a tube, we can determine whether antigen and antibody have combined by testing to see whether the complement has been fixed or not. If complement has been fixed, then we know that properly suited antigen and antibody were in the mixture. Knowing the identity either of antigen or antibody, we can identify the other.

The Wassermann Test. A common use of the complement-fixation test is the diagnosis of syphilis. It was first described by Wassermann. It serves to illustrate a complement-fixation method, which is generally applicable. It may be explained as follows: The serum of the patient, which (we shall assume) contains the syphilis antibody, is mixed in a test tube with a little of the syphilis antigen. The usual combination occurs and some or all of the complement in the patient's serum is used up, or fixed. *The difficulty is in knowing whether the complement is fixed or not,* since all of the substances, and the reaction itself, are quite invisible, and one cannot see what is going on in the test tube. One must test the mixture to see whether there is any unfixed or free complement which could *complete some other reaction if it had the opportunity.* A very simple test is to add a few red blood corpuscles *which are already sensitized* to complement with a specific red-cell cytolysin* (often called *hemolysin*). If there is any free or unfixed complement, it will combine with the sensitized red corpuscles and dissolve them. One can easily see if the red corpuscles have been dissolved (*hemolyzed*), because the suspension of red cells becomes clear and ruby-red, whereas previously it was cloudy red.

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* This antibody is easily prepared by injecting sheep erythrocytes into a rabbit. The rabbit's serum will soon contain sheep-cell cytolysins.

In practice it is the custom to destroy entirely the *varying* amounts of complement already in the patient's serum by gentle (56° C. for thirty minutes) heating (*inactivation*) and to substitute a standard, *carefully measured* (or titrated) amount of fresh, active complement from some other source, such as the serum of guinea pigs. This also enables the person doing the test to form some opinion as to how much complement remains free, since he knows how much he added originally. If *part* of it has been fixed, some of the sensitized red corpuscles will fail to be *hemolyzed*. When *all* the cells are hemolyzed, it indicates that none of the complement was fixed by the patient's serum in contact with the syphilis antigen. *It is then known that the patient did not have syphilis antibody in his serum.* The same method is employed to detect and study many other sorts of antigen and antibody.

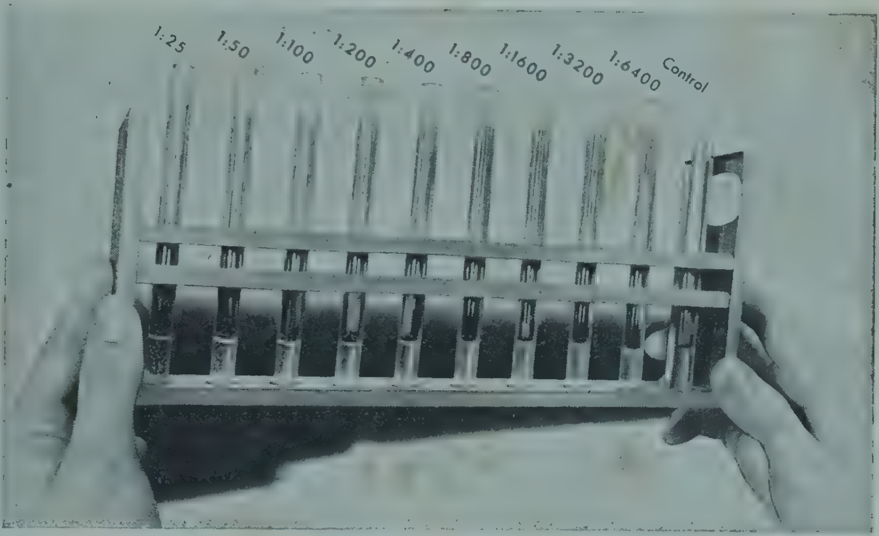

Fig. 139. Agglutination test. After incubation and refrigeration, the test is read by noting the degree of agglutination in each of the tubes. (Seminar, vol. 6.)

Agglutinins. In addition to antitoxins, cytolysins and complement, there are antibodies which cause bacteria to gather and stick together in flocks or clumps as though they were coated with some glutinous substance. Such antibodies are called *agglutinins*. Like all other antibodies, they are specific. They do not necessarily kill bacteria but aid the leukocytes by gathering the latter's prey into groups. A leukocyte, or other phagocyte cell, can engulf 50 agglutinated bacteria fifty times as easily as 50 separate ones, and in much less time.

Agglutinins are very widely used in the identification of bacteria and the diagnosis of disease. Let us assume that a patient has a severe fever which has remained undiagnosed for a week or more. We draw a little blood from a vein and allow it to clot. We then remove the clear serum and mix it, suitably diluted (1:20; 1:40; 1:80; . . . 1:2560), in a series of test tubes with, for example, a suspension of typhoid bacilli (*Salmonella typhosa*). If the patient has typhoid fever, the serum will contain *typhoid agglutinins* and the bacilli will be found in flocks or clumps even in high dilution (Fig. 139). If the fever is due to some other organism (and assuming that no antigenic components of the latter organisms are shared in common with typhoid bacilli so as to cause cross-reactions) no antibodies capable of agglutinating typhoid bacilli will be present and the typhoid bacilli will not be agglutinated. This means of diagnosing *typhoid fever* is called the *Widal reaction* after Widal, who first published upon the subject. The test as used by Widal is, however, subject to a number of serious errors and it is better to use the process of receptor analysis described later (see page 323).

Identification of Bacteria by the Agglutination Reaction. Conversely to the Widal test, in the identification of an unknown organism, sera containing various *known* antibodies* are mixed with suspensions of the unknown bacterium, and agglutination is looked for. Suppose, for example, that we have a gram-negative rod which, by its cultural reactions, we know to belong to the typhoid-dysentery group. We may, as a preliminary test, set up two series of tubes: A, containing serial dilutions of serum of a typhoid-immune animal; B, containing dilutions of serum of a dysentery-immune animal. A drop of our "unknown" bacterial suspension is added to each tube. If, after several hours, no change has occurred in the first series of tubes while the serum in the tubes of series B has caused the bacilli to fall to the bottom of the tubes in flakes or granules, we know that, since the serum in B contained only dysentery agglutinins, our unknown organism must be some species of *Shigella* (dysentery bacilli). Many other bacteria, saprophytic and parasitic, may be identified in this way. For very exact work it is advisable to use sera the specificity of which has been increased by the process of antibody adsorption previously described.

Somatic and Flagellar Agglutinins.† It will be remembered that flagella stain differently from the bodies of bacilli. This and other data suggest that flagellar substance (carbohydrate or protein or both) differs in some way from cell body, or somatic protein.* Agglutinins (which are also referred to as *receptors*) produced by motile bacilli of the typhoid-paratyphoid group and other groups are of two sorts, flagellar and somatic. One sort, the so-called *H agglutinins* (or H receptors), are engendered by, and act only against, the antigen present in the *flagella* (H antigen). With a few exceptions (*e.g.,* the genus *Proteus*), the flagella of each motile species stimulate the production of agglutinins active against the H antigen of *only* that species.

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* These antibodies are readily prepared by injecting the desired antigen into a rabbit. The rabbit's serum will soon contain the specific antibodies.

† In this discussion the student must bear in mind *constantly* the distinction between *antigen* and *antibody*.

The second kind of agglutinins, called *O agglutinins* (or O receptors) are engendered by, and act only against, the proteins present in the bodies (soma) of the bacilli. These body proteins, or *somatic proteins,* are usually not all species-specific but some of them may occur in several species of any group of closely related organisms, as previously described. Such agglutinins, being common to a group of organisms, are often called *group agglutinins.*

Receptor Analysis. The H antigens of motile bacilli may be destroyed by treatment of the bacteria with warm alcohol (37° C.). Thus, we may obtain a suspension which will react only to the O antibodies of a given serum although the serum may also contain H agglutinins. (A nonmotile variant will react in the same way.) The O proteins are not so readily affected by alcohol, but we may prepare a suspension of flagellated organisms which will not react to O antibodies, but only to H, by treatment with formalin or phenol. The flagella are stiffened and protrude from the bacilli like spines on a sea-urchin. Thus the flagella can come into contact and form *large flocculent* clumps under the influence of the H agglutinins, because the bodies of the bacilli are held apart although O agglutinins may be present. When the O agglutinins act on O antigens without the interference of flagella, *very fine granular* clumping is observed. Such preparations are of great use in diagnosis as they enable us to analyze a patient's serum with respect to the types of receptors (or agglutinins) present. The process is called *receptor analysis,* and is useful in studying disease.†

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* Recent studies indicate that what we speak of as flagella may in reality be merely threads of capsular substance. This may be, but it does not alter the utility of the concept that the flagellar (capsular?) substance is immunologically different from the somatic substance and we shall here use the concept of flagella until the question is settled.[16]

† **Antigen for demonstrating H agglutinins:** With as little agitation as possible, wash the growth of a motile strain of *Salmonella typhi* from a 24-hour-old agar slant culture with 0.85 per cent NaCl solution and dilute to an appropriate density. Add 0.2 per cent formalin and store for use. In testing, incubate the serum-antigen mixture at 37° C. for 2 hours.

Antigen for demonstrating O agglutinins: Proceed as above, using a minimum of saline solution. Add equal volume of absolute alcohol, mix and store for 24 hours at 37° C. Shake once or twice. Add sufficient saline solution to bring alco-

For example, the serum of persons recently *infected* with an organism of the typhoid-paratyphoid-dysentery group will show a characteristic marked *increase** in the amount of O agglutinins for this group. Thus, we may determine to what *group* the infecting organism belongs. If there is also marked increase in the concentration of H agglutinins, it is possible to state as a rule, but not with absolute certainty, which *species* in the group is causing the disease, since the H agglutinins are, with few exceptions, species-specific. A person *injected with a vaccine* made of killed bacteria of the group, as is often done for preventive purposes (see next chapter), after the lapse of a few weeks or months will usually show little or no *permanent* increase in O agglutinins but may show a persistent increase in H agglutinins corresponding in type to the species of bacteria injected.

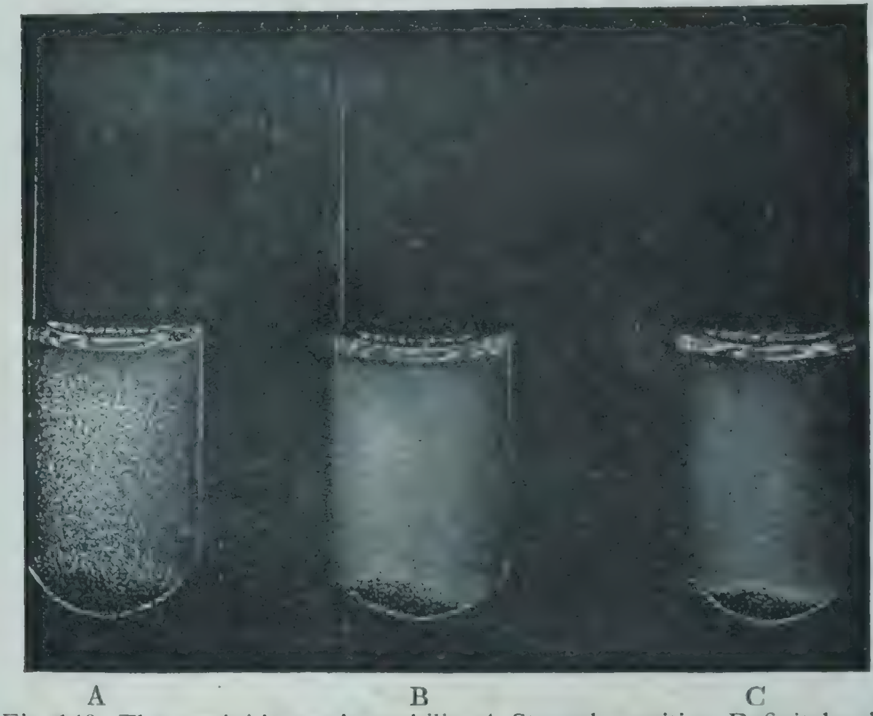

Fig. 140. The precipitin test in syphilis. *A*, Strongly positive. Definitely visible particles are suspended in the transparent medium. *B*, Weaker reaction. Fine particles are suspended in a somewhat turbid medium. *C*, Negative reaction. The medium is transparent and free from particles. (From Kahn, "The Kahn Test," Williams & Wilkins Co., publishers.)

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hol to 33 per cent concentration (1/2 total volume). Store in the cold. Keeps well. Dilute with at least 5 volumes of saline solution before use. In testing, incubate the serum-antigen mixture at 50° C. for 24 hours.

* Normal serum often contains small amounts of certain antibodies, especially agglutinins of various sorts.

Precipitins. In the agglutination reaction whole cells, like bacilli or erythrocytes or other cells, are clumped together. *Precipitins,* which seem to be very closely related to agglutinins and which are probably merely a different manifestation of the same antibodies, cause the clumping and precipitation of *invisible molecules* of protein so that a visible turbidity or flakiness is formed as described in the section on toxin and antitoxin (see page 318). Serological identification of soluble proteins of closely similar composition is thus possible, a scientific feat impossible of attainment by chemical means.

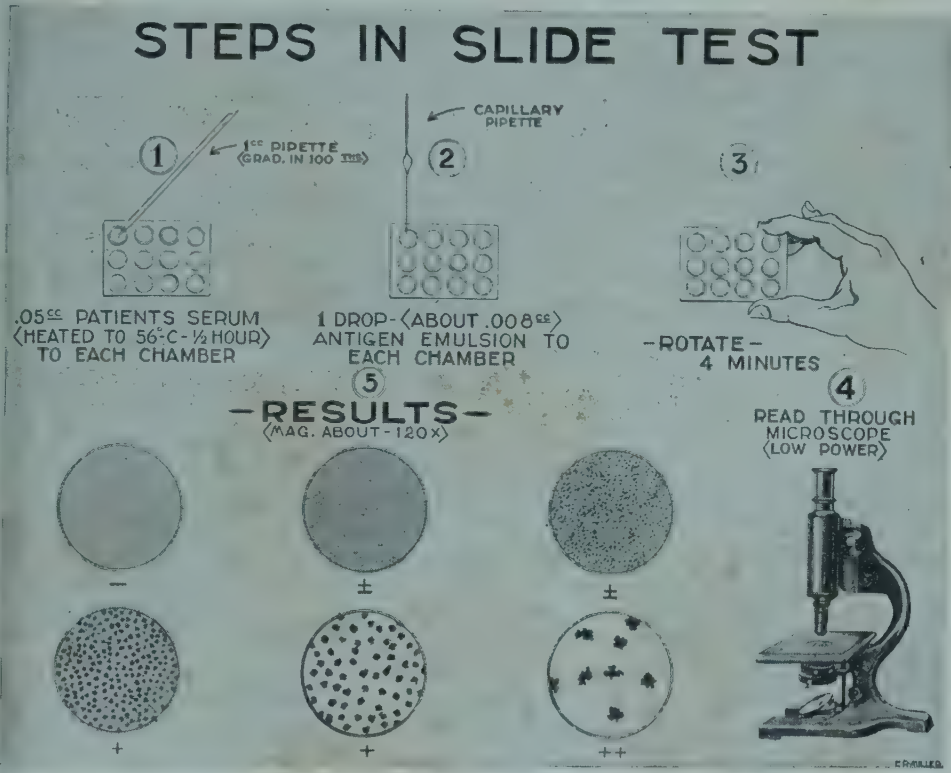
STEPS IN SLIDE TEST
①
1cc PIPETTE (GRAD. IN 100 THS)
CAPILLARY PIPETTE
②
③
.05cc PATIENTS SERUM (HEATED TO 56°C - ½ HOUR) TO EACH CHAMBER
1 DROP-(ABOUT .008cc) ANTIGEN EMULSION TO EACH CHAMBER
-ROTATE- 4 MINUTES
⑤
-RESULTS- (MAG. ABOUT - 120X)
④
READ THROUGH MICROSCOPE (LOW POWER)
−
±
±
+
+
++

Fig. 141. Equipment and procedure in the Kline slide precipitation test. (Courtesy of Dr. B. S. Kline.)

The procedure is similar to that used in the agglutination test. It is very simple and is valuable in many ways. For example, it is possible to determine whether minute blood stains are of human or some other origin, even when the stains are many years old. Pure proteins derived from bacteria and other sources can also be studied in the same way.

The Precipitin Test Applied to Syphilis. It seems to be well established that, in complement fixation tests such as the Wassermann reaction, the complement is fixed because it is *adsorbed* onto finely divided particles of precipitate resulting from an interaction between the antigen and antibody. In the Wassermann reaction, the precipitate formed is not visible. It would be a very great advantage if, instead of having to test for the presence of this invisible precipitate by adding complement and then being forced, in turn, to test for the presence of complement by adding sensitized red corpuscles, we could see the precipitate directly as in other precipitin reactions. This has been accomplished.

A specially prepared and very concentrated alcoholic antigen is used, in which the reactive substances are present in the form of large, unstable, colloidal complexes. These are brought, by proper dilution with saline solution, to a state where, in contact with syphilitic serum, they precipitate in a visible form while no precipitation occurs in the presence of normal serum (Fig. 140) *under proper circumstances.*[17, 18]

The "Slide Reaction." The so-called "slide reaction" is sometimes used as a rapid routine method in performing the precipitin test. It is also widely used in agglutination tests with a wide variety of antigens and antibody-containing sera (*antisera*). In this method a loopful or 0.1 ml. of the serum to be used, properly diluted if necessary, is placed on a glass slide. The antigen, either soluble protein such as egg white, or bacterial suspension, is immediately added by means of a loop or a measuring pipette and the serum and antigen are gently mixed with a loop or by tilting the slide back and forth. Very often the reaction is discernible with the naked eye as a granular or "curdled" appearance of the antigen; other times it must be observed with the high-dry lens of the microscope (Fig. 141).

The method is widely applicable for soluble protein antigens (precipitin tests) and for cellular antigens (agglutination tests). The reaction shown in the figure follows the procedure devised by Kline for the diagnosis of syphilis. Glass plates with depressions to hold the serum-antigen mixtures are often used.[19, 20, 21, 22, 23]

Protective Antibodies. All of the immune reactions so far mentioned have been demonstrable by test tube methods. It was mentioned that *immunity* does not necessarily result from the presence of such antibodies. Indeed, the exact significance of these antibodies for protection against disease is not always clear. When this became known scientists sought for a more exact measure of the power of the serum to protect against infection. What better method could be devised than actually to infect experimental animals (*e.g.*, mice) and give them doses of the serum to be tested to see whether they are thereby protected? This measures *protective power* directly, regardless of whether this power depends on agglutinins, cytolysins, or some still undiscovered antibody.

Such a test is known as a *protection test* and is widely used to measure the antigenic virtue of antigens and the protective power of sera. For example, suppose we have a typhoid bacillus suspension which we wish to test for antigenicity. Suppose it is to be used for inoculating soldiers against typhoid fever during maneuvers. Our best procedure is to inoculate several persons with the vaccine and then test their serum for antibodies. But shall we test for H agglutinins, or O agglutinins, or some other? We can best find out if the serum of such persons *actually protects* against typhoid infection by injecting a small amount (say 0.5 ml.) of the serum into a mouse and shortly afterward giving him an injection of live typhoid bacilli just about large enough to kill were no protection given by the serum. By varying the dosages of serum and bacilli, one may measure the *protective potency* of the serum.[24]

The protection test is of course useful in many other applications. A similar test, often called the *neutralization test,* is used in connection with filtrable viruses (see section on Viruses, page 789).

Coproantibodies. It may be that antibodies in the blood are ineffective when the organisms are not reached by them, as for example in cholera or dysentery when the organisms are confined to the lumen of the bowel. In such conditions antibodies in the feces (called coproantibodies, see page 564) may be of decisive importance.

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Chapter 17

IMMUNOLOGY AND MICROBIOLOGY

2. Production of Immunity

In the previous chapter it was pointed out that persons might become immune to certain diseases by surviving natural attacks of those maladies. The body acquires specific properties of resistance against such infections. This sort of immunity, not existent in the normal person, is acquired in the course of natural events and is therefore spoken of as *naturally acquired* immunity. In this chapter we shall speak of some other kinds of immunity especially of purposefully induced immunity, or *artificial immunity.*

Artificial Immunity. While immunity to disease may result naturally from contact with infective agents and even though subclinical or inapparent* infections often occur, with the development of immunity as a result, diseases causing immunity are all too frequently very severe and may be disabling and disfiguring or fatal. We have no entirely reliable control over natural disease.

It would be much better if we could become immune by some means which we can control and so avoid the dangers and discomfort necessarily inherent in the natural process. Furthermore, we should like to become surely and safely immune to disease early in life and not have to wait for accidental natural infection, occurring perhaps at a very inconvenient time in adult life. In addition, it is often desirable to be able to produce an immunity to certain diseases at certain definite times. For example, a person desiring to do laboratory research with yellow fever virus would like to be able to immunize himself safely and surely before starting the work, since natural infection with the virus might result fatally. So also, physicians and nurses or others working with typhoid or diphtheria patients should be immunized safely and comfortably against these diseases in time to begin their work. All this, however, is too much to expect of Nature.

In view of these needs, man, following the lead of Pasteur, has devised means of developing specific immunities "artificially" and safely. The methods involve natural processes, but are used under

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* Inapparent infections are those in which symptoms are so very mild that no special attention is paid to them. The vast majority of infections, fortunately, are of this type.

modified and *carefully controlled* conditions and are therefore called "artificial immunization." Two types of artificial immunity are used: *active artificial immunity* and *passive artificial immunity.*

Active Artificial Immunity. In active artificial immunity the patient's body is stimulated to produce antibodies by being injected with certain kinds of antigens. These are of three general types, as follows: (*a*) sterile bacterial *exotoxins,* or (*b*) sterile microbial cellular antigens (proteins and carbohydrates), or *endotoxins,* in the form of dead microorganisms, or (*c*) living, infectious microorganisms, the virulence of which has been reduced or attenuated by various procedures so that no *serious* infection results.

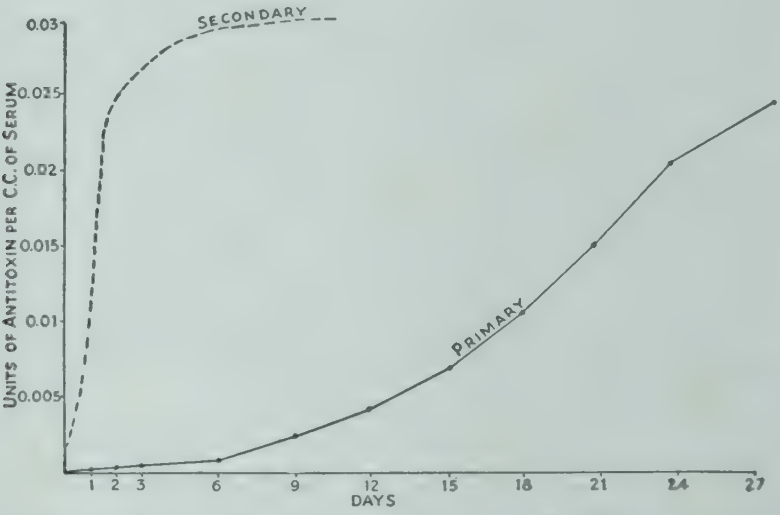


Fig. 142. Curves showing rate of antitoxin production following a primary injection and following a secondary injection. Note that after the secondary injection antitoxin production is much more rapid and extensive than after the first or primary stimulus.

Primary and Secondary Stimulus. An important fundamental principle in active artificial immunity as well as in natural immunity is illustrated by the effect of repeated doses of antigen on active immunization. In the ordinary course of life, one is constantly being exposed to repeated doses of infectious organisms such as pneumococci, streptococci, and diphtheria bacilli from carriers and inapparent infections. These repeated stimuli serve to keep one's immunity in a good state. This phenomenon is used in artificial processes also. For example, suppose that a child be given a single dose (1 ml.) of diphtheria antigen such as alum toxoid. In two weeks, his blood, tested by appropriate methods, shows very few antibodies. After four weeks, however, his blood is found to contain a satisfactory amount of antitoxin (say 0.5 unit per ml.). The development of immunity has been slow! A year later this is found to have diminished to a very low figure (say 0.001 unit). This diminution of antibody concentration in the serum is very common.

After time has reduced the effect of the first antigenic stimulus, let us give the child a very minute *second* dose of toxoid (say 0.005 ml.) and test his blood for antitoxin at short intervals. A surprisingly rapid and extensive response is noted. Whereas, after the *first* or *primary stimulus* given a year before, response was slow (even to a large dose of toxoid; 1 ml.), response now to $\frac{1}{200}$ this dose, when given as a *secondary stimulus,* occurs in a few hours and the child may be found to have 1 or more units of antitoxin per ml. of blood inside of two or three days (Fig. 142). In practice the secondary stimulus is often referred to as a "booster dose."

It is as though the body cells, having once had an "antigenic experience," are more "expert" at forming antibodies of this particular sort and do so with great facility whenever called upon. It is upon this very rapid reactivity that the child's ability to withstand the disease may largely depend. It explains, in part, why resistance to disease and permanent concentration of demonstrable antibodies in the serum are not necessarily related. Each time the specific antigen comes into contact with the child's tissues, the latter react like a well-trained "flying squad" of guardsmen and repel the invader before it can gain any foothold and cause disease.[1, 2] The principle of the primary and secondary stimulus is a generally applicable one and should be borne in mind. It is not restricted to toxins but works equally well with living or dead bacteria, egg white, and other antigenic substances.

Immunization with Toxins. This procedure will be readily understood by those who have read the preceding chapter. Cultures of toxin-producing bacteria, like *C. diphtheriae,* are made in broth and, after sufficient growth, are passed through porcelain filters which remove the bacteria. The *filtrate* (broth passing through the filter) contains the exotoxin. This may be injected hypodermically (under the skin) in from 1 to 5 very minute doses at weekly intervals into the persons to be immunized and eventually (usually after two to six weeks) their blood will be found to contain antitoxin which protects them from the toxin in question. This procedure or a modification of it was formerly used in immunizing children against diphtheria. There is considerable danger, however, and many fatal accidents occurred due to overdoses of toxin even when a little antitoxin was mixed with it.

Toxoids. Soon after its discovery it was observed that diphtheria toxin is quite unstable and rather rapidly undergoes spontaneous deterioration, especially if exposed to a warm climate or sunlight. The toxin is altered by warmth and light, and also by such substances as formaldehyde, until it is no longer poisonous. It is then spoken of as *toxoid*. Far from losing its value in such a condition, it has been found that toxoid, in spite of being nontoxic, nevertheless retains its specific antigenicity and engenders antibodies which neutralize the toxin. In order to avoid the dangers attendant upon the use of toxin, toxoid came into general use for active immunization against diphtheria. The same considerations apply to other toxins, such as that of the tetanus (lockjaw) bacillus, etc.

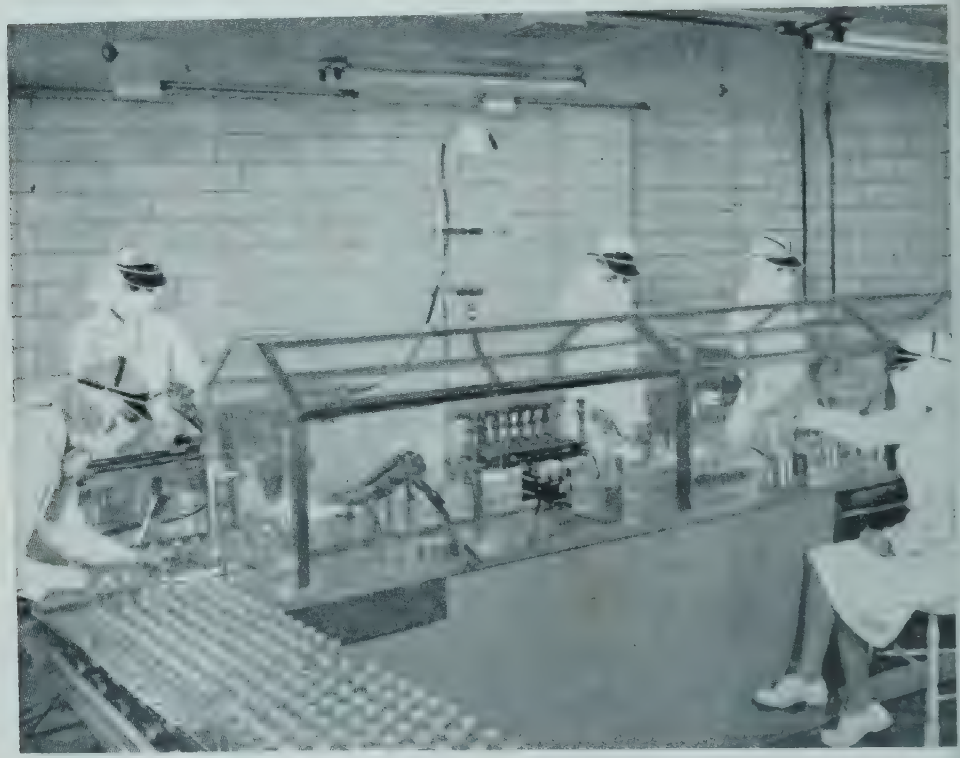


Fig. 143. Sterile filling machine for bottling parenamine amino acid solution, for parenteral injections. The entire filling apparatus is enclosed under plexiglass and moving bottles are constantly subjected to bacteria-killing ultraviolet rays. (Courtesy of Frederick Stearns & Co.)

It was later found possible to improve such antigens still further. The addition of alum to broth containing the toxoid *precipitates* the latter and it may then be collected, concentrated and purified to some extent. Alum-precipitated toxoid is highly effective and only one or two injections are needed as compared with three injections of fluid toxoid antigen. Similar discoveries have been made concerning other toxins used in active artificial immunization, notably the toxins of scarlet fever and tetanus.[3] Kendrick and Eldering[4] have adapted the principle to bacterial vaccine against whooping cough (see page 780). It is now common practice to mix several toxoids, as diphtheria and tetanus toxoids, as well as bacterial antigens. The immunization is just as effective and the number of separate injections is reduced.[5, 6]

Action of Adsorbed Toxoids. It is important to understand why alum-precipitated toxoid is more effective than plain, fluid toxoid. When the latter is injected under the skin, the soluble material and fluid are quickly adsorbed by the body and destroyed and eliminated inside of a few hours. The *antigenic stimulus* is very transitory, and repeated injections are necessary. If the toxoid could be held *in situ* for several days, being released continuously little by little into the body, the antigenic stimulus would be prolonged and continuous and there would be no need for further injections.

This is accomplished by means of the alum-toxoid. The toxoid is adsorbed and held on the surfaces of the alum precipitate, which is insoluble. The alum precipitate remains *in situ* where injected undissolved, releasing its *adsorbed* toxoid slowly, little by little, giving the patient a prolonged and continuous antigenic action which is highly effective.

This principle is not confined to diphtheria immunization but is of broad significance. The existence in the body of an antigen, living or dead, which persists there and maintains immunity by its continuous antigenic action, is sometimes spoken of as "premunition."

Immunization with Dead Bacteria. A second procedure of active, artificial immunization is very similar to the foregoing except that, instead of removing bacteria from the broth in which they have grown and using their excreted growth products (*e.g.,* exotoxin), the bacteria themselves are used. For convenience, greater yield, and avoidance of foreign matter, they are usually cultivated on agar and removed to physiological saline solution which is then heated to about 60° C. to kill the bacteria. A very minute amount (0.25 per cent) of phenol, tricresol, or other antibacterial agent is added to insure sterility. A modern apparatus for filling bottles with sterile material under aseptic precautions for use as vaccines, drugs, serums, etc., is seen in Fig. 143.

Such suspensions of killed bacteria are frequently referred to as vaccines but they are correctly termed *bacterins;* the term "vaccine" is properly restricted solely to the immunizing agent against smallpox.

Methods of killing bacteria and viruses for vaccines by means of ultraviolet irradiations seem to give good results.[7]

The method of employing bacterins is so effective in preventing typhoid and paratyphoid fever that their use against these diseases in the United States military forces has long since become a matter of regulation. The principle of the secondary stimulus is now made use of by the U. S. Army in connection with these bacterins. After a soldier has received an ordinary course of three weekly inoculations as a primary stimulus, his resistance is maintained by single, annual, intradermal injections of 0.1 ml. of bacterin. These cause little or no reaction, are quick, inexpensive and easy and, above all, effective in reinforcing waning immunity.[3]

The hypodermic injection of carefully controlled doses of suspensions of killed staphylococci is also much used to increase resistance of persons susceptible to boils, styes, etc., which are usually due to staphylococci. The use of *Hemophilus pertussis* bacterins in preventing whooping cough is of great value also.[8]

Antigenic Strains of Bacteria. An important principle in the use of bacterins has been demonstrated especially in connection with typhoid and pertussis immunization. We have noted that bacteria, when invading the body, often undergo variations which increase their virulence. Such changes may involve the acquisition of capsules or certain obscure alterations in physical or chemical make-up or both which aid them in invading the body. Upon isolation from the body and cultivation on artificial media, they lose these peculiar properties and revert to a more or less saprophytic state. In this state, while still capable of infecting under favorable circumstances, they are less irritating or virulent (or antigenic) and may, indeed, prove quite ineffective as bacterins. It is important, therefore, to select, for bacterins, freshly isolated organisms of known antigenic value.

In the case of *Hemophilus pertussis* the organisms when freshly isolated from a pertussis patient and in a properly *antigenic* state are of the smooth, encapsulated type and are highly virulent for mice. This condition of the organisms is spoken of as "phase I." In the case of *Salmonella typhi*, the antigenic strains are smooth, highly virulent for mice and exhibit on their surfaces a certain component called the "Vi antigen" in enhanced degree. Such strains stimulate production of *protective antibodies* which may be distinct from agglutinins, etc. Organisms devoid of these special properties may stimulate the production of certain antibodies, as agglutinins or cytolysins, but this does not always mean that they produce effective immunity or protective antibodies to disease since, as has been pointed out, immunity and test-tube-demonstrable antibodies are not synonymous.

Immunization with Attenuated, Living, Infectious Agents. The third method of artificial active immunization consists in actually infecting the person to be immunized, with the desired organism which is of greatly attenuated or modified virulence.

There are at least three means of lowering the virulence of pathogenic organisms so that they can be safely used to induce active, artificial immunity. These are *animal passage*, *desiccation*, and *cultivation on special media* or *under special conditions* such as abnormally high temperature.

Animal Passage. An early use of the principle was tested by Pasteur who attenuated the organisms of swine erysipelas (*Erysipelothrix rhusiopathiae*) by injecting them into rabbits and then recovering them and treating swine with them. The swine often became immune after a mild attack of the disease. This method is not used extensively. Animal passage often greatly enhances virulence of microorganisms.

Another form of this process is very well illustrated by the method of preparing material for the classical vaccination against smallpox, although the organisms causing this disease are not bacteria. They belong to the group of viruses. However, the principle involved is the same whether viruses or bacteria are used.

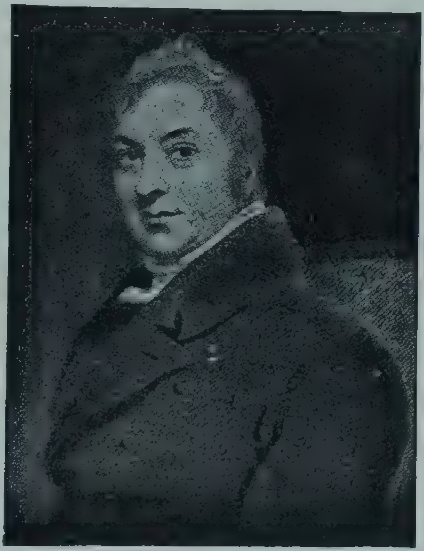
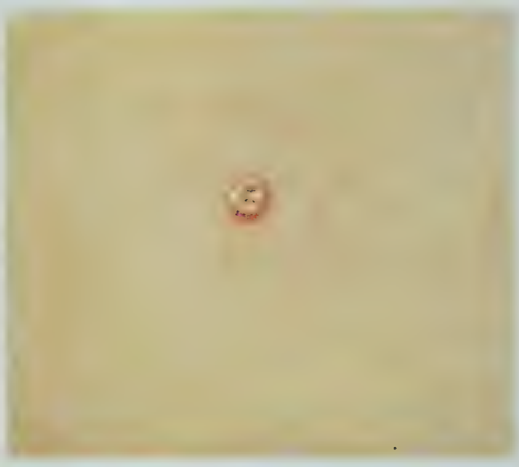


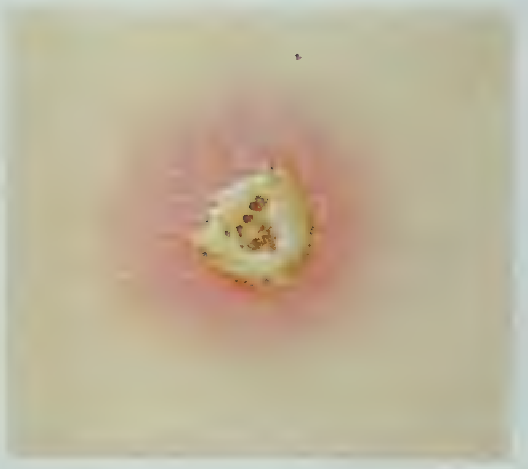
Fig. 144. Edward Jenner, 1749–1823. (From the painting by Sir Thomas Lawrence.)

Smallpox virus causes *smallpox* in human beings. Cowpox virus causes cowpox in cattle and can infect man as well. Cowpox, however, is quite mild in man and never results fatally, and the pustules remain localized. Cowpox is regarded by some as a modified form of smallpox. However that may be, it causes only a very mild harmless infection of human beings.

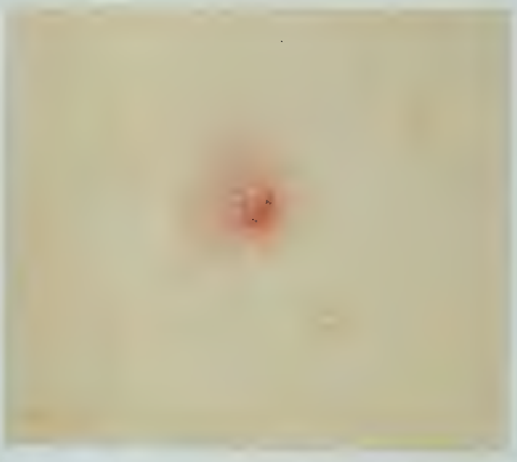
In 1798 Jenner (Fig. 144), then a country doctor but later a very famous British scientist, observed that many dairy workers associated with cows having cowpox did not succumb during epidemics of smallpox. Experimenting, he found that if some of the serum or lymph from the pustules on the udder of a cow with cowpox were scratched into the arm of a human being, a very mild disease (*vaccinia*) resulted, with the formation of a single, localized, poxlike lesion



Appearance of a normal "take" three or four days following vaccination.



By the ninth or tenth day the reaction has reached a maximum, following which the redness fades and a scab begins to form.



In those cases in which immunity is high a prompt and very mild reaction occurs.

Fig. 145. (Courtesy of Sharp & Dohme, Biological Products, 1947.)

which soon healed, leaving a distinctive scar (Fig. 145). *Smallpox* never developed in persons after infection with *cowpox*. The person thus safely became immune to smallpox through infection with cowpox, cowpox virus being, as we have said, possibly smallpox virus in a modified form, or at least a closely related organism.

Vaccine virus, as used today, is prepared by scratching cowpox or smallpox virus into the shaved and disinfected skin of a calf. When the pustules are "ripe" the lymph is collected from them and put up in glass tubes ready for use (Fig. 146).

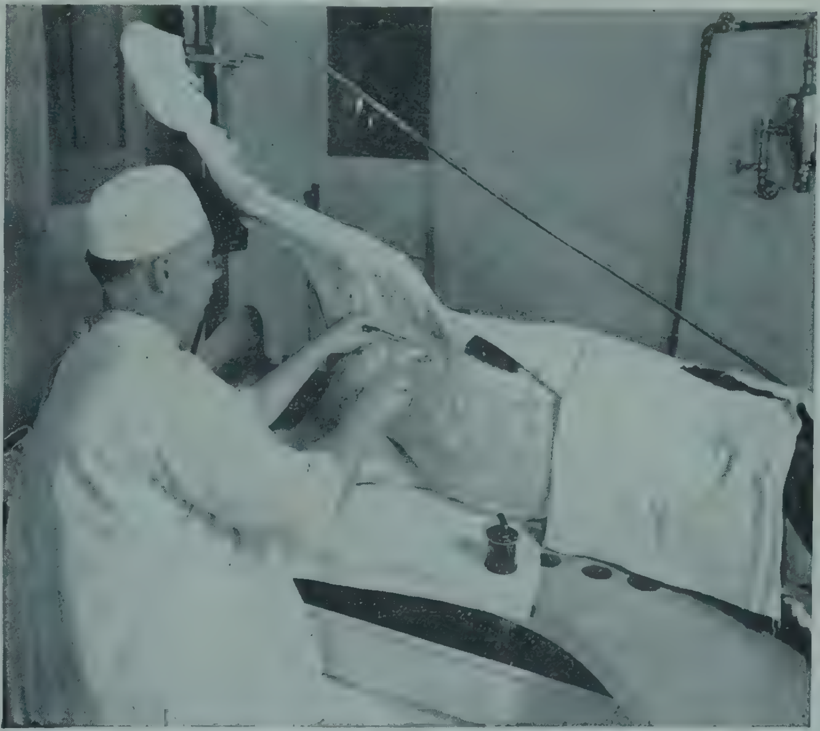


Fig. 146. Collecting lymph containing vaccinia virus.

Its potency and cleanliness are carefully controlled by the National Institute of Health at Washington, D. C. Its value is made evident by experience and by a glance at Figure 147.

The importance of vaccine is well-illustrated by an episode in 1947 in New York City in which the disease was introduced by a traveler from Mexico City. It began to spread in and from New York. The immediate and effective mobilization of over 6,350,000 people in a mass vaccination may have prevented a holocaust such as would probably have wiped out 1.5 to 2 million people in two or three weeks if the episode had occurred during the unvaccinated middle ages. Only twelve cases and two deaths occurred in the New York episode. One of these deaths was the original patient from Mexico City.[9]

Desiccation. Immunization Against Rabies. Pasteur's name is immortalized in the term "Pasteur treatment" for *rabies* or *hydrophobia* ("mad-dog bite"). The process is not really a curative "treatment" but is a course of immunizing injections with the living but attenuated virus of rabies*; or it may depend on the phenomenon of *interference*, described on page 802. If started soon enough after the bite of the infected dog, resistance (or interference) develops from the injections before the virus from the dog-bite can cause disease.

For years Pasteur experimented with rabbits, dogs and guinea pigs (one of the many brilliant illustrations of the value of animal experimentation) until he felt morally certain that his method of attenuating rabies virus was safe and effective. It consisted in passing the virus from an infected dog to the brain of a rabbit and then from rabbit to rabbit to establish a uniformity of infectivity for rabbits that resulted in an *attenuated* virulence for man. Strangely, it has a *maximum* and fixed virulence for rabbits, and Pasteur called this virus "virus fixé." The potency of the virus was further reduced by drying or dilution (Högyes method) and was injected in increasing doses.

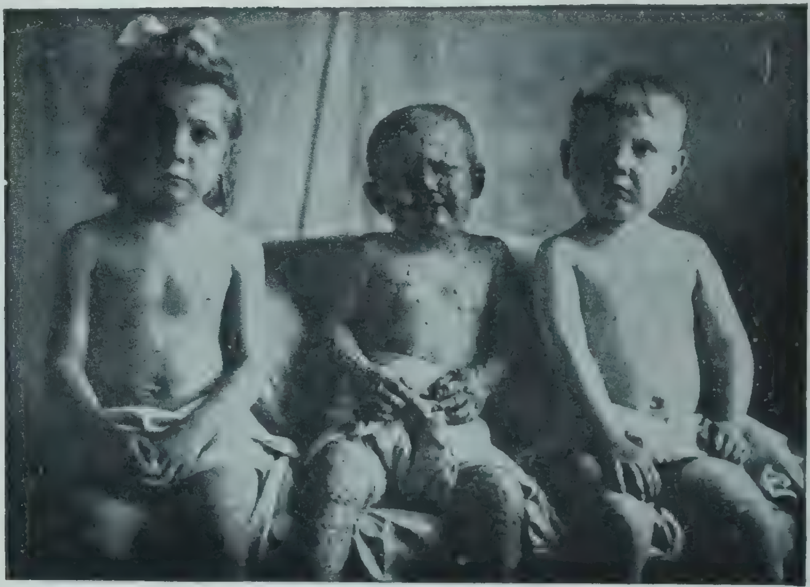


Fig. 147. Children of one family who were brought to the Municipal Hospital of Philadelphia with the mother and father, who had smallpox. The child in the center had been considered too young to be vaccinated. The other children had been vaccinated a year before; they remained free from the disease, although for several weeks they lived in the wards of patients with smallpox. (From Welch and Schamberg, "Contagious Diseases," Lea and Febiger, publishers.)

Pasteur's first human immunization against rabies, in 1885, was an extremely dramatic event and marked an epoch in the progress of the war on disease. Indeed, Pasteur is referred to as "the father of immunology." His experiments on rabies have been well described by Vallery-Radot, Pasteur's grandson.[10]

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* In modern "Pasteur treatment" a killed virus is used.

Today immunization of dogs against rabies is accomplished by means of brain and spinal cord tissues containing virus inactivated by various means. In the Semple method it is treated with phenol. Kelser and Schoening,[11] and Leach[12] have shown the value of chloroform-treated vaccine. Webster[13] and his coworkers have demonstrated that virus inactivated by ultraviolet light has considerable value. The great difficulty with preparations in which the virus is killed is that so little actual virus is present that the antigenic stimulus is small. Very large doses have to be given. A method being given favorable consideration in 1948 was the cultivation of the virus in chick embryo, which yielded a fairly good product. Another process of purifying brain virus by benzene-ether extraction gave promise of a more effective immunizing agent.[14]

Cultivation in Special Media. Immunization Against Tuberculosis. A method for immunization against tuberculosis has been known for some years. The procedure is known as the "Calmette process" and the attenuated culture as "B. C. G." (Bacillus Calmette-Guérin). The value of the method is still in question, but the data available seem promising. All are agreed that the method is *safe*. The process consists of a series of injections of *living* tubercle bacilli, the virulence of which has been reduced by cultivation of the organism on certain media containing bile. This procedure probably induces the development of a stable variant of low virulence. Similar variants may easily be produced in other culture media. This is the only important use to date for immunization of human beings with bacteria, attenuated by cultivation on special culture media. The process is discussed further in the section on tuberculosis (see page 753).[15, 16]

Attenuation by Cultivation at Unfavorable Temperatures. Although this method is not much used at present, its discovery and demonstration by Pasteur constitute an episode of great dramatic interest in the history of bacteriology. The principle was first used with *Pasteurella avicida,* the cause of fowl cholera. It was later developed in connection with studies of anthrax which, in the nineteenth century, decimated the sheep flocks of France and still does so in some parts of Europe. Following numerous preliminary experiments by the tireless Pasteur, on variants of *Bacillus anthracis,* cultures of the organisms were prepared by cultivation at temperatures of from 39° to 43° C. (optimum around 35° C.) which caused them to lose some of their virulence (and also, often, their power of spore formation).*

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* The method has since been shown to vary in result so that neither loss of spores nor cultivation at 42° C. should be assumed to have deprived these organisms of their dangerous properties.

The immunizing agent finally adopted by Pasteur consisted of broth cultures of these attenuated strains. The first great public demonstration of the value of anthrax immunization gave the indomitable Pasteur some uncomfortable hours, because his ideas had been very unfavorably regarded by many and his experiments were very closely watched. He injected a certain number of sheep with his attenuated organisms. After a suitable period had been allowed for antibodies to develop, these animals, as well as an equal number of normal, unprotected animals, were to be publicly inoculated with large doses of fully virulent anthrax bacilli. This experiment has been dramatized in a motion picture. The test injections were made on May 31, 1881, at Pouilly le Fort. There were many skeptics in the audience when the injecting began.

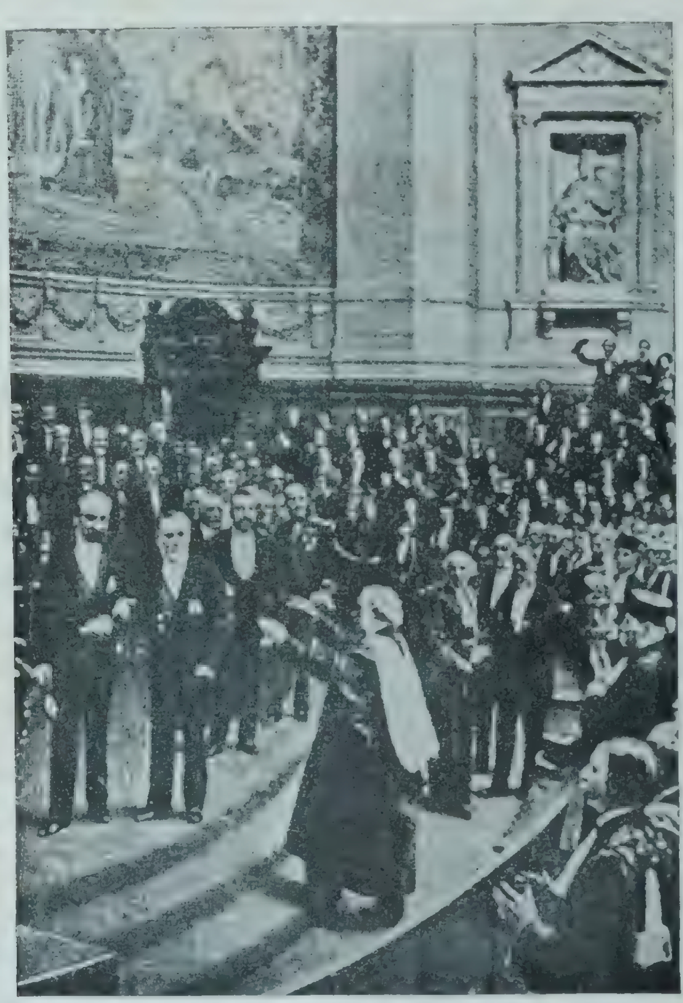

Fig. 148. Pasteur's Jubilee 1892. The painting represents Lister greeting Pasteur at the Sorbonne.

A day or two later, when Pasteur arrived at Pouilly le Fort, "the carcasses of twenty-two unvaccinated sheep were lying side by side; two others were breathing their last; the last survivors of the sacrificed lot showed all the characteristic symptoms of splenic fever (anthrax). All the vaccinated sheep were in perfect health."*

An interesting review of the 1948 status of these and other active immunization procedures has been prepared by Muckenfuss, Bell, Holt, Long and Top.[17]

Passive Immunity. An *important point* to remember in connection with all active immunity, whether due to natural disease or to the artificial use of antigens, is that when antigens first gain entrance to the body a period of time, possibly two to six weeks, may elapse before the antibody response is evident. This interval is often called the *latent period.* If the antigen gaining entrance to the body be diphtheria toxin from diphtheria bacilli infecting the throat, this lapse of time is of great importance if the patient is suffering from a severe, clinical case of the disease. Obviously treatment with bacterins, toxoids or attenuated organisms would prove futile. The toxin is formed by the bacteria so very rapidly that the patient may die before his body cells can possibly manufacture enough antitoxin to neutralize the toxin. A single hour may mean the difference between life and death.

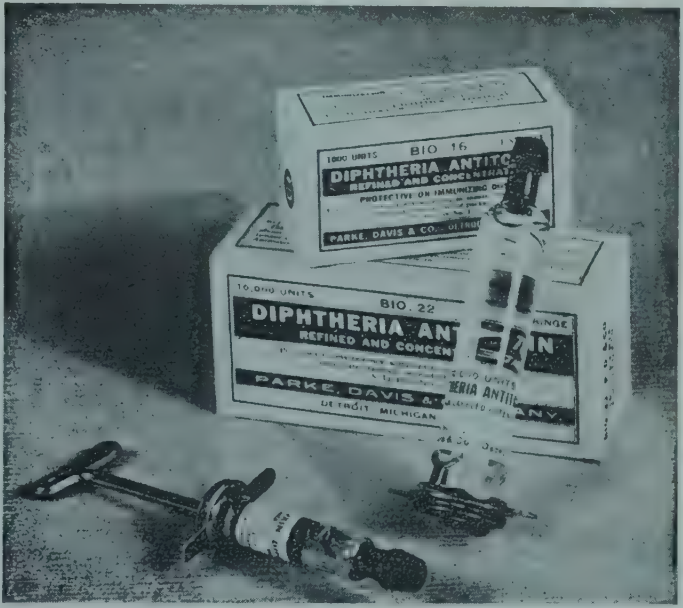

Fig. 149. Commercial syringe—package of antitoxin.

What an advantage it would be if the desperately needed antibodies could be immediately supplied in generous excess from some outside source to tide the patient over his emergency! These antibodies are available in large quantities in the form of serum taken from a previously immunized animal. Such antibody-containing serum can be purchased at any well-stocked drug store in sterile syringes all ready for injection (Fig. 149). It is like a loan of life from the great reserve bank of nature to avert an immunological overdraft! Unfortunately not all infections are amenable to serum therapy. For example, good serum is not available for the treatment of staphylococcus infections or tuberculosis. Such organisms seem to have little power to engender protective antibodies in such amount that the serum has value in passive immunity. They are said to be poor antigens.

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* From "The Life of Louis Pasteur," by René Vallery-Radot, reprinted with permission from Doubleday, Doran & Company, Inc.

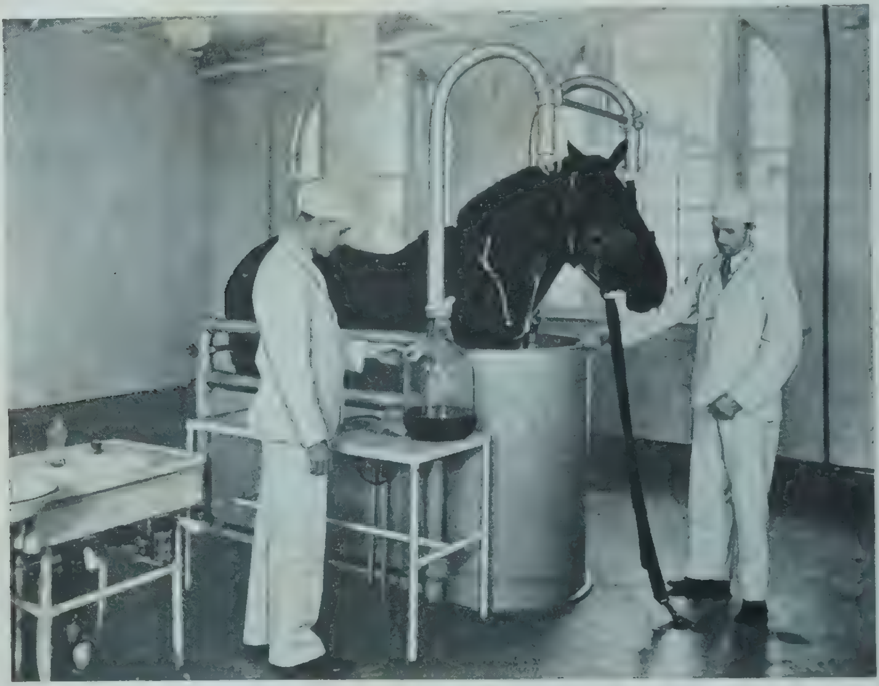

Fig. 150. Production of antitoxin from horses. About eight liters of blood are taken at each bleeding. Potassium oxalate prevents clotting. (From The Physicians Bulletin, Nov.–Dec., 1944, Eli Lilly and Co., Indianapolis, Ind.)

Commercial Manufacture of Antitoxin. Antitoxin-containing serum, be it diphtheria or tetanus antitoxin, or any other sort, is taken from strong, clean and healthy horses or other animals which have previously received a series of harmless injections of the proper antigen. The first doses of antigen injected into the horses are small, but increase in size as the animal becomes more and more immune and his blood contains more and more antibody. At the end of several months a quantity of blood is drawn from the animal with no more pain than the bite of a fly (Fig. 150) and the serum is removed after the formation and contraction of the clot, or the oxalated plasma may be used (Fig. 151). The serum or plasma contains the antibodies in large amounts. Various methods of extracting and concentrating the antibodies are used so that the human patient may receive large amounts of antibody is as small a dose as possible.

Transitory Nature of Passive Immunity. When patients receive injections of such antibody-containing serum, they become *immediately* immune, not through any active antibody formation on their own part, but by the *passive reception* of ready-formed antibodies. Their immunity is, therefore, said to be *passive immunity*. Passive immunity is *transitory* and all the injected antibodies disappear within a few days or weeks. One can become *permanently* immune *only* through the *active* process, either *natural* or *artificial*.

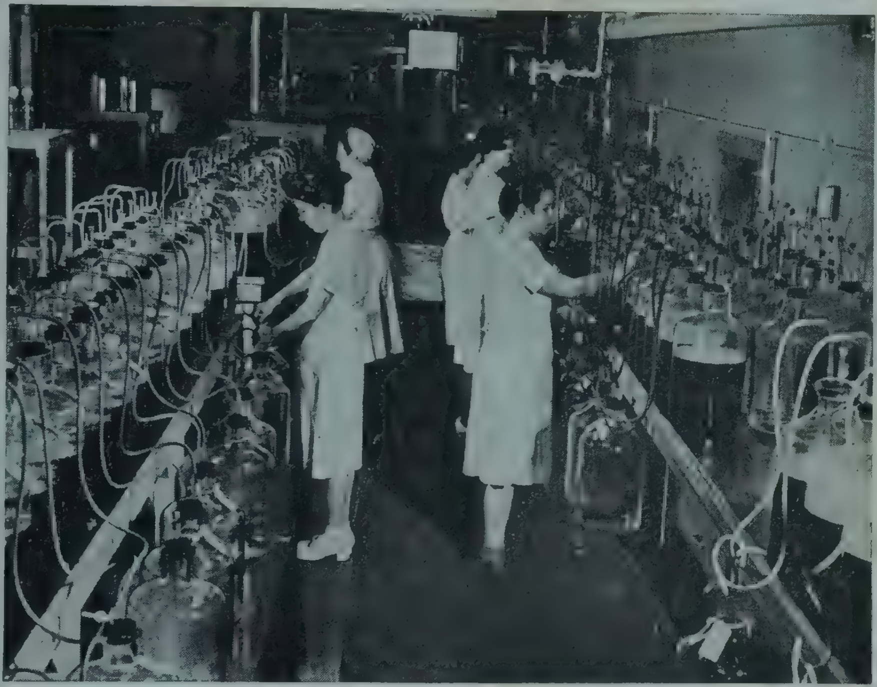

Fig. 151. Production of antitoxin from horses. The cellular portion of the blood settles to the bottom of the bottle, leaving the supernatant plasma which is siphoned into sterile containers. (From The Physicians Bulletin, Nov.–Dec., 1944, Eli Lilly and Co., Indianapolis, Ind.)

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Chapter 18

IMMUNOLOGY AND MICROBIOLOGY

3. Allergy

We now come to an immunological paradox. The process of active immunization, artificial or natural, may indeed cause an animal or person to become more resistant to a disease, yet so alter his physiological state that the antigen, though originally harmless, now affects him as would a violent poison. He is said to be in a state of *allergy* (*allos* = change; *ergon* = action) or *hypersensitiveness* with respect to the antigen involved. The immune state and the allergic state are closely related, equally specific, and involve similar if not identical physiological mechanisms.

The entrance of an antigen into the body parenterally, regardless of source or nature, be it bacteria, toxin, serum, milk, oyster, plant juice or what you will, not only engenders circulating antibodies against that antigen, but also alters the body tissues so that a second injection of the same antigen, given *about three weeks* after the first, produces an immunological response which is much more rapid and vigorous than the first. This response may be perceptible only as an increase in antibody production (see secondary stimulus, page 330), which is part of the defensive system of the host, or as ability of tissues to bind and hold the antigen at its site of entrance so that it does not spread further although it may damage the tissue locally where it is held.[1] These reactions are beneficial to the host. On the other hand, reactions of allergy may be so violent and widespread as to injure seriously or even kill the host. The mechanisms involved are not entirely clear.

Anaphylaxis. While the term allergy or allergic reaction is used to include all reactions due to hypersensitiveness, many exclude from the term one particular reaction which does not occur naturally and is almost unique in its properties. This may be illustrated as follows: Assume that some animal, as dog, rabbit or (preferably) a guinea pig, has received an injection of 1 ml. of some foreign protein, say horse serum. After a variable period of around three weeks this animal is found hypersensitive to a second injection of horse serum, and will exhibit a very striking response called an *anaphylactic* reaction (*ana* = against; *phylaxis* = protection. Contrast with pro-phylaxis).

Within one minute after intravenous or intraperitoneal injection of even a minute secondary dose of the antigen, the animal (if a guinea pig) becomes uneasy and scratches at its nose, coughs, and is evidently embarrassed for air. Gagging movements occur, and the animal gasps for breath. Urination and defecation take place, the animal falls on its side and ceases to breathe. Death may supervene within a few minutes. If the attack is not fatal, recovery is often abrupt and seemingly complete within an hour or two and the animal will not exhibit hypersensitiveness for some days or weeks afterward. The animal is said to be *desensitized.*

Most of the symptoms are believed due to contraction of smooth muscle fibers, large amounts of which are present in the lungs, thus constricting the air passages. The fibers are also present in the intestines and bladder. Reductions in temperature and other signs are probably due to the release of a histamine-like poison, often referred to as H-substance, by the tissues involved. In rabbits, dogs and other animals the picture varies, probably due to differences in anatomical location of smooth muscle. In pregnant animals abortion often occurs because the uterus consists largely of smooth muscle. In dogs there is much damage to the liver and this alters the clinical picture markedly in them.

The mechanism in anaphylaxis appears to be fairly clear. It seems to depend on the fact that the tissues contain antibodies *fixed to the cells which produced them.* Then an antigen-antibody reaction takes place immediately on or in those cells, with varying results, the nature of which depends on the species of animal, the kind and location of the cells, the violence and extent of the reaction, and whether or not the second dose of antigen was partly neutralized by reacting with circulating antibodies before it reached the sensitized tissue cells.

In human beings typical anaphylaxis rarely occurs, but many other manifestations of hypersensitiveness are seen. A common one is "hay fever." All are closely related but there are certain differences which may justify considering allergic reactions as a separate group of phenomena—distinguished from anaphylactic reactions. For example, anaphylaxis is an artificially induced condition; allergy spontaneous. Desensitization to anaphylaxis is easy; in allergy it is often difficult or impossible. The nature of the antibodies in allergy differs from those in anaphylaxis. In anaphylaxis the antibodies are probably precipitins. This is shown by the phenomenon called *passive anaphylaxis.*

Passive Anaphylaxis. If the serum of an immunized animal containing precipitins is injected into a normal animal, then the tissues of the normal animal adsorb the precipitins after a few hours and are ready to react just as though the normal tissues had produced the antibodies. The animal will show typical anaphylaxis. This is *passive anaphylaxis*.[2, 3]

ALLERGY

If anaphylaxis be excluded as an artificial and unique phenomenon, then the term allergy may be used to include all other phenomena of naturally occurring hypersensitiveness. While animals may exhibit some forms of the condition the best-known manifestations of allergy occur in man.

Basically, the mechanisms of allergy and anaphylaxis appear to be similar. Allergy is regarded as a reaction between antigens (with respect to allergy they are called *allergens*) and antibodies (in allergy called *reagins*). Allergens are sometimes classed as *inhalants* if, like ragweed pollen, they gain entrance to the body by inhalation; *ingestants* if eaten, and *injectants* if injected.

As in anaphylaxis the allergic reaction occurs in the tissues with the liberation of a poisonous H-substance which resembles histamine. In allergy, however, the main sites of reaction (called "shock organs") are: the conjunctivae, the mucosae of the nose and gastrointestinal tract, the skin and the brain. Thus, the manifestations of allergy in man are very different from those of anaphylaxis in animals in which smooth muscle of deeper organs is involved. Allergic symptoms may often be combatted by the use of antihistaminic drugs, of which a goodly number are now on the market.[3a]

Unlike anaphylaxis, it is very difficult to produce passive allergy, and after an allergic reaction there is no period of nonreactivity as there is following anaphylactic shock.

A particularly interesting point about allergy is its apparent relation to inheritance. The hypersensitiveness itself does not appear to be inherited, but the tendency to become hypersensitive does. In order to manifest this tendency persons must come in contact with an allergen. They are very easily sensitized. Persons with this inherited tendency are said to be *atopic*. They often show very marked allergic tendencies, developing hay fever, hives and numerous other manifestations very readily. They are desensitized with difficulty (see page 346). Reagin may be demonstrated in their blood in considerable amounts. Non-atopic persons do not so readily develop hay fever, etc.; less commonly have such severe attacks, if any; do not have circulating reagins in the blood and are more easily desensitized. Various forms of allergy may be referred to by the *allergen,* or by the clinical manifestation: thus hay fever, food allergy, cosmetic rash or dermatitis, etc. Here we shall use common terms and may discuss only a few general types of reaction.[4, 5]

Types of Allergic Reaction. *Pollen Allergy.* The pollen of many plants, among them ragweed, grasses, roses and others of which the pollen is light enough to float around in the air, are capable of producing hypersensitiveness in some persons. The protein (allergens) which the pollen cells contain, landing on the oral and respiratory mucosae of these persons, sets up a hypersensitive condition of these tissues so that when the person later inhales more of the same pollen, the tissues of the mucous membrane lining his nose and throat become irritated, swollen, and edematous with serum or lymph, and all the symptoms of a severe "cold" may develop, due to the severity of the reaction against the pollen. This condition is very well known and is commonly called "hay fever" or "rose fever." A person may *sometimes* be desensitized by receiving a series of small, hypodermic injections of the proper pollen if he is not an atopic individual.[6, 7, 8]

Food Allergy. By an analogous process, a person may become sensitized to a certain food, for example, strawberries or codfish. If for some reason, such as a slight gastrointestinal irritation, lesion, or other disturbance, some of the protein of the food gets past the stomach or intestinal wall and into the blood in an *undigested* or partially digested condition, the result is the same as if the protein had been injected or inhaled. The next time, and perhaps every time, the person eats strawberries or codfish, he may react more or less violently. Usually under these circumstances the allergic reaction manifests itself in the form of a rash or blotches on the skin which itch persistently. These are commonly known as "hives." In other cases, the victim may become violently ill and exhibit nausea, vomiting, intestinal irritation, and perhaps other symptoms. Such persons may often be desensitized, as in pollen hypersensitiveness or anaphylaxis, by receiving one or more injections of the protein of the appropriate food.

Bacterial Allergy. Bacteria as well as viruses and other microorganisms entering the blood stream or tissues may sensitize certain cells of the body to their protein as well as stimulate antibody production. The relation between antibodies and the reactivity of the sensitive tissues is not clear. In many instances the tissues of the skin seem highly sensitive, a condition readily demonstrated by intradermal injection of the antigen, which results in a local redness and swelling. This may be due to the presence of the antibodies in the cells of the skin.

Whenever an infectious disease becomes *subacute* or chronic, all of the body cells may become sensitized to the bacterial protein, and give rise to various symptoms and little-understood reactions. A person who has been infected with the tubercle bacillus remains in an allergic condition to the organism, an important consideration in regard to

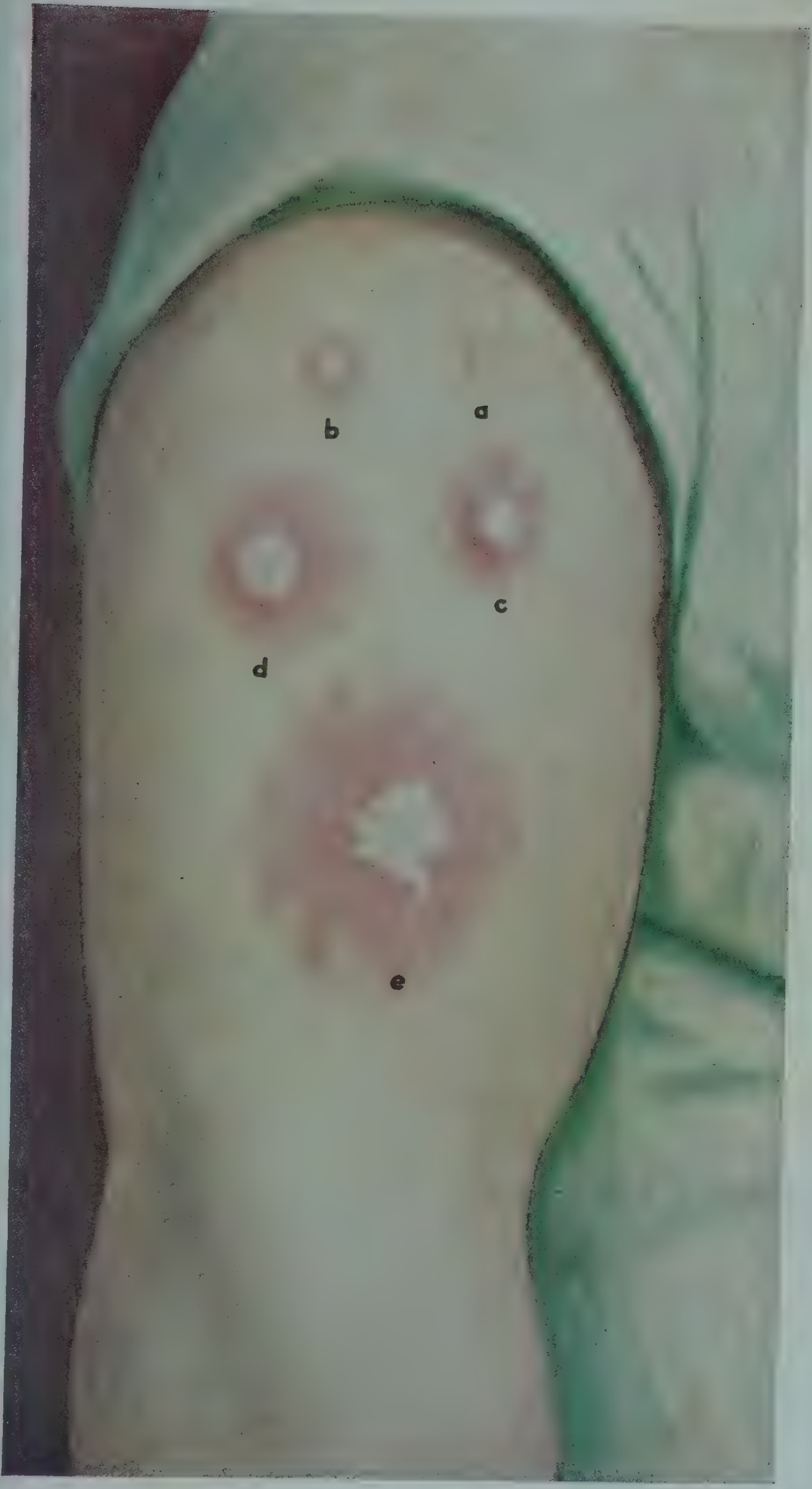

Fig. 152. Reactions after intracutaneous injection of allergenic extracts: *a,* negative (control); *b,* doubtful; *c,* slight; *d,* moderate; *e,* marked. (Kolmer and Tuft.)

his resistance to the disease. Skin tests for allergy to tubercle bacilli (called *tuberculin tests*) are of great value in the study of tuberculosis as will be pointed out later (see page 751). Allergy, therefore, plays an important part in infectious diseases. Other diseases of this chronic nature are syphilis, undulant fever, swine erysipelas and leprosy.

Dermal Reactions. In hypersensitive persons as well as in hypersensitive animals the skin often exhibits a power to bind and localize the antigen. In such localized reactions necrosis of the skin may follow a marked edema.

Arthus Phenomenon. Among these localized skin reactions is the phenomenon described by Arthus. If a rabbit receives injections of some foreign protein, say monkey serum, over a period of several weeks, the later injections cause ulcers and sterile abscesses to occur at the site of inoculation. These develop very quickly, and there is no anaphylactic reaction, indicating that the antigen is held in the tissues around the site of the inoculation so that it does not reach other cells of the body.

Schwartzman Reaction. If a rabbit be injected *intradermally* with an antigen, say a sterile, filtered broth culture of certain bacteria, the site of the intradermal injection, although apparently unaffected after the *first* injection, will react violently, with a hemorrhagic and necrotic lesion, when similar material is injected *intravenously* twenty-four hours later. It is as if the tissues at the site of the first injection removed the material later injected into the blood stream, and bound it, with deleterious results locally. The exact mechanism of this reaction is not clear. It is not entirely specific.

Dermal Tests for Hypersensitiveness. The skin of a person or an animal hypersensitive to any given protein will develop a localized, inflamed area where a small quantity of that protein has been scratched or injected into the skin. This local reaction is simply another manifestation of the allergic condition, but it is extremely useful in determining what protein is causing a given patient's trouble.

In tests for food or pollen sensitiveness, sterile solutions of various foods or pollens which would be likely to be involved, are introduced *into* (not under) the skin, either by scarification or injection, and the results observed. A number of "likely" substances are usually tested at the same time (Fig. 152). The one causing the most severe reaction is probably the cause of the patient's trouble.[6]

Serum Sickness. After the injection of serum for the treatment of disease, such as diphtheria or tetanus, there often occur undesirable reactions due to sensitivity of the body cells to the proteins in the serum. The antitoxin has nothing to do with the reaction, since normal horse serum will cause the same reaction. These reactions may occur in persons not known to have had previous injections of serum. In these instances the reaction is delayed, sometimes for several weeks, probably until sufficient antibodies have accumulated in the tissues to react with the antigen which may still persist in the body.

Serum sickness appears in various forms ranging from acute and serious illness accompanied by collapse and dyspnea (difficulty in breathing) coming on a few minutes after the injection in already hypersensitive or atopic people, to milder, delayed forms such as arthritis, hives or extensive rashes.

As these reactions are most likely to occur in patients who have asthma and other symptoms of allergy, *i.e.,* hypersensitive or atopic persons, investigation is usually made of this point before serum is administered. If the serum is to be given intravenously, a preliminary skin test for hypersensitiveness to horse serum may be made, as described below. If the test is positive, the patient is *desensitized,* as previously described, by giving very small preliminary doses of the serum.

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Chapter 19

MICROORGANISMS AND DISEASE

We have already seen that in the course of evolution microorganisms appeared which were capable of growth in or upon the tissues of plants and animals and that by such growth they cause disease. Here we shall deal with the principal properties which appear to confer on microorganisms their pathogenic quality, the properties of the animal host (the infected or parasitized animal) which render it susceptible or resistant to infection, and a few other general aspects of the host-parasite relationship, especially those involving microbial parasites.[1, 2]

Except for a dozen or so genera, bacteria and related microorganisms are quite harmless and many are, indeed, entirely indispensable to the continued existence of higher forms of life.[3] With those harmless and useful microorganisms that are engaged in maintaining the fertility of the soil and acting as scavengers we have, in our everyday life, little concern and can control them only in the most limited sense. Certain species are of industrial use or are important as causes of decay, spoilage, rot and fermentation, and we therefore either exploit them and induce them to serve us or devise means of killing and avoiding them. With respect to those few species which have taken on the habit of parasitism and disease production, man has for many years been engaged in the most intensive study and has expended incalculable amounts of money, time, energy and human and animal life in efforts to combat or exterminate them.[4–8]

What Is Disease? An exact definition of disease is difficult of formulation. We may say that disease is a departure from normal. But what is normal, and how great must be the departure before it falls into the category of disease? Possibly the simplest plan for the present is to fall back on the generally accepted meaning of the term disease and leave such academic arguments to the lexicographers. Disease, we shall say, is any visible or sensible, *harmful* departure from the "normal" condition of the body. Such conditions often result from the entrance into the body of organisms which are able to multiply in, and to damage, body tissues.

Parasitism and Pathogenicity. A distinction must be drawn between *parasitism* and *pathogenicity*. A wholly effective (but hypothetical) parasite is one which is wholly nonpathogenic and yields absolutely no benefit to its host. A differentiation between this relationship and commensalism might prove difficult. Such a parasite does no damage, stirs up no reaction on the part of the host against itself and so is left undisturbed to live out its ungrateful existence. Most *actual parasites* (as distinguished from commensals), however, occasion *some* damage to their host, although perhaps little. Some do so much damage that they soon kill the source of their livelihood and in this respect are inept parasites—possibly beginners in the evolutionary scale of parasitism. They are effective pathogens but not highly adapted *parasites.*

On the other hand, pathogenicity does not always involve parasitism. For example, certain organisms like the bacillus causing lockjaw or tetanus (*Clostridium tetani*) cannot invade normal tissues, but live only in dead (necrotic) material, such as might occur in a crushed foot, causing disease only by their toxins. The organism of food poisoning, *Cl. botulinum,* likewise cannot multiply in the tissues or on the body but produces a poison in foods outside the body which becomes effective (lethal) when swallowed.

Pathogenicity Is Fortuitous. Those not familiar with microbial disease sometimes conceive of microorganisms as predatory, purposeful creatures producing poisons and invading the tissues for the sole purpose of causing harm as though that were to their benefit in the sense that a tiger, snake or spider benefits from killing its prey. They are sometimes thought of as being endowed with special powers for injury. We have just seen that excessive injury to the host will also result unfavorably to a parasite. The greatest advantage to the parasite results from unnoticed parasitism, not from cannibalism! Damage to the host is purely accidental and not necessary to the parasite.

Most soil-inhabiting microorganisms lack the physiological properties which might enable them to multiply within a host. If we consider man as the host, many microorganisms of the soil find the body temperature too high. Even though the temperature be suitable many cannot live in contact with organic material. Others require cellulose as a source of carbon. Still others require a different *p*H, and so on. Some find conditions in or on the body which will permit their growth but they cannot resist the action of the leukocytes. The first requisite to successful continued parasitism, then, is ability to live in or on the host, and this without stirring up a reaction with which the parasite cannot cope. This ability seems to have developed as a process of evolution.

Mutual Adaptation. The process of adaptation between host and parasite must be thought of as a mutual one, requiring many generations of natural selection of the most adaptable. Bacterial generations are sometimes very short, a few minutes or hours; human generations are long, about twenty-five years. Thus bacteria can become adapted to growth in contact with human tissues before man acquires species or racial or active resistance to invasive bacteria.

We may imagine that a few bacteria are just beginning the process of adaptation, as they stir up a violent reaction (acute disease) nearly every time they come into contact with the host and begin multiplying. Gonococci and pertussis bacilli are examples. The host, on the other hand, has not yet become adapted to them. Others are better adapted; the host, through many generations of contact, seems to have become more used to having them growing within him, and chronic disease ensues. Syphilis, tuberculosis and leprosy may be thought of as examples. In still other instances adaptation may be complete and might be represented by the apparent commensal relationship between the harmless organisms of the throat, skin and intestine, and the host. In still other instances the human host and dangerous pathogens like the typhoid bacillus (*Salmonella typhi*), pneumococci and meningococci become mutually adapted and live together for years with no evidence of disease. This is a common situation and will be discussed later as the *carrier state*.

Factors in the Occurrence of Disease. In discussing disease due to infection we have to consider several factors: (1) the means by which the parasite enters the body (portal of entry); (2) its means of maintaining itself there and multiplying (aggressiveness; virulence) and its effect upon the host (pathogenicity); (3) the means by which the host overcomes the parasite (host defenses); (4) the portal by which the parasite leaves one host to reach another (portal of exit); (5) the factors which affect survival of the parasite outside the host (resistance of parasite to external environment); and (6) the mechanisms which effect transmission from one host to another (vectors of disease).

Not all of these factors will be discussed in this chapter. Some are treated more in detail in other places in this volume in connection with specific diseases.

1. *Portal of Entry*. If the skin be kept intact, no ordinary bacteria can get through it.* But if any slight cut or scratch exists, then bacteria can get into the tissues. The thin membranes about the eye (conjunctivae), in the nose and throat and in the genitalia are less able to withstand invasion of some bacteria than the tough outer skin, and infections sometimes begin in such situations.

The portal by which an organism enters the body is an important factor in determining the occurrence and kind of disease. Certain bacteria can establish themselves in the body only by first coming into contact with the respiratory tract, others only through contact with the intestinal lining and so on. Thus, dysentery bacilli (*Shigella*

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* Some few microorganisms are said to be able to penetrate the intact skin.

dysenteriae), rubbed over the hands, would cause no infection while, if swallowed, they might produce a fatal disease. On the other hand, *Neisseria gonorrhoeae* might be swallowed without harmful effect but if rubbed in the eye or genitalia could cause gonorrheal infection of the mucous membrane.

The skin and mucous membranes of the body are inhabited by large numbers of bacteria, some of them highly pathogenic under some circumstances. They are usually held in check by phagocytic tissue cells and leukocytes. The secretions of the body are also important defenses against these bacteria. Some of the body fluids, such as perspiration, gastric juice and the vaginal secretion, are so acid as to destroy many bacteria. Even though not killed by the acidity, they may be removed mechanically by the secretions. Thus, bacteria caught on the sticky surfaces inside the mouth, nose or lungs are moved upward by the cilia of the epithelial cells lining the air passages and then removed in the mucous and saliva by coughing, swallowing or sneezing (Fig. 153). Leukocytes also are found in the secretions and are often seen in gram-stained smears of nasal or vaginal mucus, or saliva, sometimes with bacteria which they have ingested.

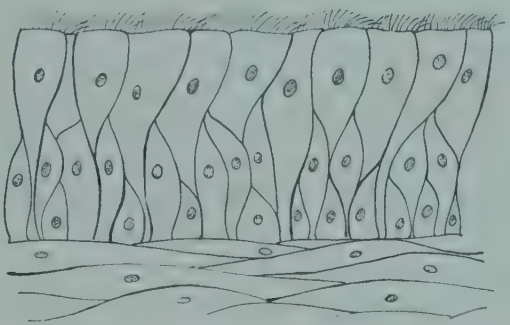

Fig. 153. Section through the wall of a bronchus, showing the ciliated epithelial cells. These cilia are in constant motion carrying dirt and bacteria upward toward the mouth. The walls of the trachea and nasal sinuses are similarly lined with ciliated epithelium.

2. *Aggressiveness.* Once gaining access to the body, saprophytic bacteria are usually immediately ingested and destroyed by leukocytes or destroyed by certain enzymes, or both. Pathogenic bacteria, however, frequently have the power to excrete enzyme-like substances which kill and drive away leukocytes. Such substances are called *leukocidins* and *anti-opsonins,* respectively. If the microorganisms avoid or destroy the leukocytes and certain other tissue cells which phagocytize, they may have an opportunity to grow in the body and they may or may not excrete poisonous waste substances called *toxins.*

Toxins. With reference to microorganisms, a toxin is a poisonous protein or protein-like substance. The toxins of bacteria are of two sorts: exotoxins and endotoxins. *Exotoxins* are poisonous waste products excreted by the bacteria into their environment. They stimulate the production of antitoxins.

Endotoxins remain within the bacterial cell or are closely attached to it. Some endotoxins may actually be the cell protoplasm itself. Most bacterial endotoxins are proteins or are substances associated with proteins. Some are carbohydrates of the capsule as in pneumococci and type B influenza bacilli. Just how they act in the body is not clear. Some, as the toxins of *Clostridium botulinum* and certain staphylococci, are harmful when swallowed. Other toxins, like that of diphtheria bacilli, can be taken by mouth with impunity, but if injected or absorbed into the blood in very tiny doses may cause death. Some appear to have an affinity for nervous tissue or heart muscle, kidney, etc., and cause damage to those tissues only, or principally. Some of these toxins are much more potent than cobra venom.

Virulence. The ability of the bacteria to invade and grow in the body tissues and injure the body is spoken of as virulence. Virulence may conveniently be regarded as made up of two factors: toxigenicity and aggressiveness.

$$\text{Virulence}\begin{cases}\text{toxigenicity.}\\ \text{aggressiveness (or vegetative power when in the body).}\end{cases}$$

Obviously, each of these factors is dependent upon, and the result of, other properties, and the effect upon the host is dependent upon a balance between his own resistance, adaptability, and the virulence of the bacteria.

As the microorganisms grow, they often undergo variations, in response to the conditions of the environment in the host, becoming more adapted to the host, and better able to multiply within it. For example, they may acquire capsules which protect them. They may then proceed to grow into the lymph spaces, spreading widely through the tissues. Some, however, the diphtheria organism for instance, remain entirely localized, killing tissues locally and excreting toxin. Others may grow in the blood stream and then we have the condition called *bacteriemia, septicemia* or "blood poisoning." Having gained entrance by a favorable portal (*i.e.*, a tissue favorable to their growth or maintenance*), they may be carried by the blood and localize at various other points in the body, *e.g.*, liver, spleen, bone marrow or lymph glands of the intestine, causing what are called secondary abscesses or secondary foci of infection, or metastatic infections.

Carriers. Sometimes persons who recover from contagious bacterial disease remain immune although still carrying the infective agent about with them. Such persons may spread the microorganisms abroad for days, weeks or years. This represents an effective mutual adaptation. They are called "carriers," and it is largely by them that certain diseases are disseminated. We shall have occasion to mention carriers in discussing various infectious diseases, so that the student should fully realize their importance at this point.

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* Maxcy terms this the "tissue of predilection."

Spreading Factors. *Host Defenses.* Some organisms show a marked tendency to spread through the tissues once they gain a foothold. This is illustrated in erysipelas, syphilis, actinomycosis, etc. This progressive spread through, or invasion of, a single continuous field is to be distinguished from mere mechanical transportation from one distant part of the body to another by the circulating blood.

Resistance to spread of microorganisms in tissue depends on several known factors and probably on a number of others unknown. Among the known factors are (*a*) *phagocytic cells,* as leukocytes; (*b*) *fixed tissue cells* which tend to grow around a focus of infection (such as tubercle or carbuncle) and encompass it within a retaining wall of fibrous tissue; (*c*) *clotting of blood* in capillary vessels (or liberated by hemorrhage) around the infection, so that the venules and tissue spaces are blocked by clots; (*d*) *hyaluronic acid,* a viscous, mucous-like substance filling intercellular space and blocking the advance of organisms; and (*e*) mechanical factors of *edema* (swelling).

Virulent organisms may overcome these defenses in the following ways:

(*a*) Some organisms produce poisons (toxins) which kill leukocytes. The *leukocidins*[9] of staphylococci and streptococci are good examples. (*b*) Others produce toxins which kill and destroy the cells of fibrous and other tissues in retaining sacs. This seems to occur in progressive tuberculosis, actinomycosis and perhaps syphilis. (*c*) Virulent streptococci, such as those causing scarlet fever, erysipelas and "blood-poisoning" (septicemia) produce an enzyme called *fibrinolysin*[10] which digests the fibrin in clots and presumably enables the organisms to enter the blood stream (septicemia) and invade the local tissues (as in erysipelas). Many of these also produce a tissue-digesting enzyme, *histase.*[11, 12] (*d*) Several species of organisms produce the mycolytic enzyme *hyaluronidase*[13] which can digest the intracellular mucus, hyaluronic acid. Theoretically it should aid microorganisms producing it to progress through tissue spaces or mucous plugs. Injected intradermally with bacteria, however, pure hyaluronidase tends to remain in situ, and is relatively ineffective as a spreading agent unless aided by pressure such as that provided by the volume of fluid injected into the tissues. The spreading effect then extends *only so far as the local area affected by the distension of the tissues by the fluid.*[14] If hyaluronidase be applied with an infective agent to scratches on the surface of the skin, where there is no *increased pressure,* there is no increased spread or invasion by the organism.[15, 16, 17]

The hyaluronidase produced by some organisms may interfere with their own virulence by digesting their own mucous-like capsules.[18] Such mucoid coatings, either those formed by mucoid growth or, as shown by Olitsky,[19] those applied artificially, offer a real protection to invading microorganisms against antibodies, phagocytes, etc. Hyaluronidase production by an organism is therefore not necessarily always entirely advantageous to the bacteria.[20]

Many of the changes which occur in the host as a result of infection are favorable to him but without much effect on the parasite. For example, a diphtheria patient may become able to neutralize the toxin of the diphtheria bacillus by producing antitoxin, yet the bacilli may continue to live in his throat after recovery. The patient becomes a "carrier."

Other reactions of the host are unfavorable to the bacteria and then the latter may be killed. In such processes certain tissues and the leukocytes may become more adept at phagocytizing the bacteria. Also, certain tissues produce antibodies which are discussed elsewhere in this book. Other host defenses have already been mentioned: mechanical barriers as skin and ciliated epithelium, acid secretions; race; species, etc.

Koch's Postulates. It is not always possible to be certain that the microorganisms isolated from a given disease lesion or from pus, blood or feces are the cause of the observed disease condition. Many harmless bacteria are found growing in feces, sputum and ulcerating wounds, and some would not grow there unless some other diseased condition existed first. Such adventitious organisms are called *secondary invaders*. To prove that a certain microorganism is the cause of a given disease often requires the most painstaking and careful study.

The question as to the etiological relationship of various bacteria to specific diseases was a very live one long before the time of Koch and there was much loose discussion and profitless argument regarding many bacteria and their relation to disease. When Koch established the pure-culture technic it became possible to apply exact methods to the study of the etiology of disease. He was very conservative in stating the relationships of any given organism to any particular disease.

His ideas on the subject were crystallized largely by his studies of the relationship of tubercle bacilli (*Mycobacterium tuberculosis*) to tuberculosis. Koch, like others before him, observed the bacilli in the lesions of animals dead of the disease. But he was not too ready to believe that he had discovered the cause of tuberculosis because he found the organisms present in the lesions of tuberculosis. Might not this bacillus appear in the tissues merely accidentally because the animal, being so ill, is too weak to resist its invasion? Might it not be merely a relatively harmless opportunist? Might it not represent contamination with a common saprophyte? Koch, involved in a discussion of the problem, finally stated what he believed to be the evidence necessary to prove an organism to be the cause of a disease. The evidence consists of four postulates, generally called *Koch's postulates* today, and they are, essentially, as follows:

1. The organism must be associated with all cases of a given disease and in logical pathological relationship to the disease and its symptoms and lesions.
2. It must be isolated from victims of the disease in pure culture.
3. When the pure culture is inoculated into susceptible animals or man, it must reproduce the disease. Many such inoculations into man have been made on courageous volunteers. In others, accidental infections have occurred which have provided long-wanted evidence. The value of animal experimentation is here very evident.
4. It must be isolated in pure culture from such experimental infections.

Even today the etiological relationship of some bacteria to diseases which they are thought to cause has not been established firmly on the basis of Koch's postulates. A notable example is the relation of so-called *Mycobacterium leprae* to leprosy.

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Chapter 20

TRANSMISSION OF DISEASE

Facts of fundamental importance which must be borne in mind when considering disease transmission are (1) that, although they have a very limited motility in fluids, microorganisms cannot fly or climb about of their own volition. However, they may be carried for many miles in rivers and lakes or as passengers on dust particles or other objects. Many bacterial and mold spores float about in the air unattached. For effective transmission from host to host most microorganisms depend on various extraneous moving agents for transportation. (2) A great difficulty for many delicate pathogens is that conditions in the world outside the body are too harsh. This is the penalty of extreme parasitism. Drying is fatal to some, such as meningococci, gonococci, and syphilis spirochetes. Exposure to sunlight quickly kills others, such as the tubercle bacillus. Others cannot live in natural bodies of water or in the soil. (3) With the exception of anthrax, none of the infectious diseases of man is produced by organisms which form very heat-resistant spores.* For this reason infectious pathogenic bacteria are easily killed by disinfection processes. (4) Furthermore, not only are infectious agents much restricted in the modes of travel available to them, but they must find a suitable portal of entry into a host at the end of their journey, and (5) the host must, in addition, be a susceptible individual.

Types of Transmitting Agency. There are two general types of disease transmission. Each type of transmission may be accomplished in several ways. For convenience we may list these as follows:

1. Mechanical, by:
   (*a*) fomites†
   (*b*) dust infected from any source
   (*c*) droplets of saliva or nasal secretion
   (*d*) feces, and materials contaminated therewith, such as drinking water, milk and other foods
   (*e*) hands

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* Gas gangrene and tetanus (caused by organisms of the soil getting into wounds) are not included as infectious or contagious disease.

† Fomites are objects contaminated by persons harboring infectious organisms; for example, hand hangers in buses, doorknobs, money, dishes, pencils, handkerchiefs, bed linen, etc.

(*f*) direct contact, as in the venereal diseases, or in nursing patients with discharging wounds, ulcers, etc.

2. Biological, by
   (*a*) biting insects
   (*b*) the feces of insects
   (*c*) the bodies, feet, etc., of insects.

Many diseases may be transmitted by several of these methods; others are restricted, under natural conditions, to only one or two methods.

MECHANICAL TRANSMISSION BY VARIOUS OBJECTS

Fomites. It is obvious that any object having live, infectious bacteria upon it may serve to transfer the bacteria from one person or place to another, causing transmission of disease. Thus, soiled bed linen or clothing, towels and wash cloths, handkerchiefs, eating utensils, pencils and similar objects are dangerous after having been used by persons suffering from diseases of the intestinal tract (*e.g.*, typhoid fever or dysentery), of the skin (*e.g.*, erysipelas, boils, fungus infections), or of the respiratory tract (*e.g.*, diphtheria, scarlet fever and pneumonia). Venereal diseases may likewise be transmitted by

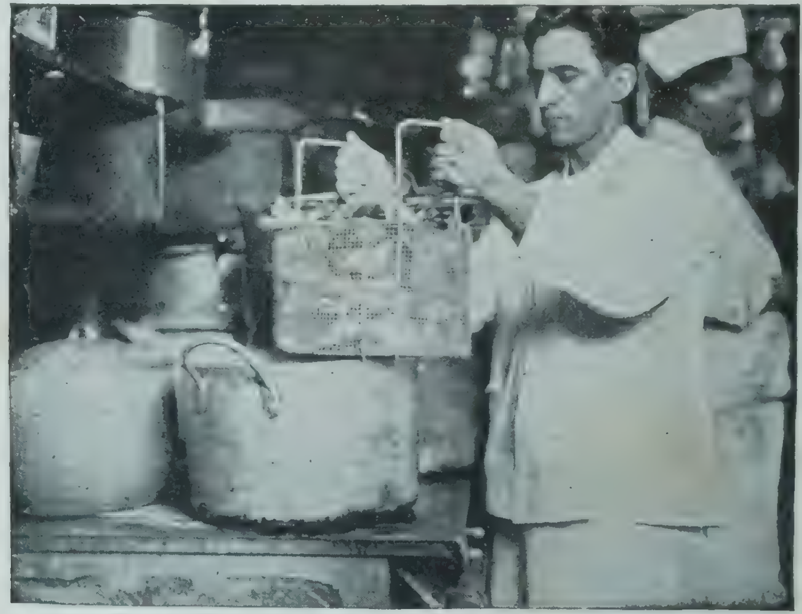

Fig. 154. Scalding after dishwashing—the safe way. (Photo by Lewis Hine. Courtesy of Cleanliness Institute.) While very fine (and expensive) dish-washing machines are available which can turn out sterile dishes,* the method shown is available to everyone, even campers in the woods.

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* If *properly* used!

these objects, although this is rare. Public toilet seats, drinking cups, hair brushes, etc., are always to be avoided.

The more recent the contact of fomites with infectious sources, the more likely are they to transmit disease. This is especially true of the diseases due to very fragile pathogens like gonococci, meningococci, pertussis bacilli and influenza bacilli. Organisms recently removed from a patient seem to be much more highly infectious and dangerous than those cultivated on artificial media. Some reasons for this have already been given (see page 169).

It is clear that to avoid the mechanical transmission of disease, objects like clothing and dishes, likely to be contaminated with discharges from a patient or carrier, should be carefully handled, segregated and boiled or steam pressed, or rinsed in solutions of disinfectants, or thoroughly sunned. Restaurant proprietors who have the well-being of their patrons in mind, either carefully scald all dishes after washing them (Fig. 154) or, after thoroughly washing them in *hot,* soapy water, rinse them in clean water containing *at least 100 parts per million* of available chlorine, and dry by drainage. The odor of chlorine around a lunch counter is a favorable sign. Other disinfectants without taste or odor are now widely used (see Chapter 11).

Sanitization of Eating Utensils. The whole problem of sanitization of public eating utensils and eating places is receiving public discussion[1, 2] and thorough scientific study by progressive health departments and agencies such as the United States Public Health Service, the American Public Health Association, the National Sanitation Foundation at the University of Michigan and the School of Hygiene and Public Health at Johns Hopkins University. Problems involved are: detergents, properties and performance; bacteriological methods (see below); usefulness and effects of disinfectants; tests for concentration of disinfectants in wash water; education of persons responsible for sanitation, and of the public; methods of examining utensils bacteriologically, and so on.

Examination of Utensils. Methods for measuring and controlling the amount of bacterial contamination of dishes, etc., are not exact. Many technical problems are involved and are under investigation.[3] Most of the methods at present favored for bacteriological examination of eating utensils center around the *swab-rinse technique* which involves the use of a sterile swab made on a wooden applicator, with cotton. This is moistened in a bland fluid (water or buffered saline solution) and used to wipe a certain prescribed area of the utensils within a few hours after they have been washed and dried. The swab is then shaken thoroughly in a small known volume (10 ml.) of sterile saline solution in a vial. Within a few minutes dilution plate counts are made of the bacteria in the fluid. From the numbers of colonies obtained an estimate is made of the degree of contamination on the dishes, etc. Commonly, a minimum standard of 100 organisms per utensil is recommended. Time and temperature of storage, humidity, dust, insects, handling, are factors affecting the numbers of organisms surviving on the dishes. A standardized method was outlined by a Committee of the American Public Health Association in 1944.[4] This specifies formulae for media, solutions, area swabbed, method of swabbing, cultural details, etc. Dilution plate counts may be made directly of the rinse water in dish-washing machines. That current dish-washing practices, as measured by a swab-rinse technic prescribed by the United States Public Health Service,[5] are not satisfactory is clearly evident.[6] The horrors of the soda fountain and cafe are none the less real because sometimes hidden!

There is no exact method of measuring the amount of disease spread by dirty dishes and eating utensils in restaurants. Probably we all have a good deal of immunity to the bacteria so transmitted. However, from an esthetic viewpoint one does not like to feel that a little saliva from previous patrons is being included, gratis, with his meal.

Paper Dishes. The use of paper cups, dishes and eating utensils is the best step toward eliminating the sanitary evils of public glass and chinaware and metal spoons and forks. Not only is expensive dishwashing equipment, with its noise, sloppiness and heat eliminated, but labor and fuel costs are reduced, breakage costs are trifling and esthetic and sanitary standards enormously improved. Bacteriological studies of paper used for containers and tableware show that the processes used in their manufacture result in a product with negligible content of microorganisms. Of these none is pathogenic.[7-10]

Transmission by Hands. Milk supplies and the food in any kitchen may become infected from the hands of careless milkers, dairymen or cooks who are carriers of infectious microorganisms, including several causes of dysentery. Many pathogenic bacteria grow very well in milk and other non-acid foods. Washing the hands after defecation or urination is a partial safeguard against transmission of intestinal disease in this way, but careless and ignorant persons are often very lax in this respect. Pasteurization of milk and the thorough cooking of foods are other safeguards. Persons who handle foods for restaurants or institutions, as well as dairy workers, should be required by law to pass bacteriological examinations and are so regulated in many communities, but enforcement is difficult and expensive.[11] There is danger from enteric and respiratory infections in food handlers, but very little from venereal diseases unless in a very active stage. The practice of holding and shaking hands doubtless transmits many pathogenic intestinal and respiratory organisms.

Transmission by Droplets of Saliva, Mucus, etc. The secretions of the nose, throat, mouth and lungs, all combined to some extent with saliva, constitute one of the most formidable vectors of disease. Pneumococci, streptococci, meningococci, diphtheria bacilli and tubercle bacilli, as well as other organisms of respiratory disease, are thus transmitted. Saliva alone is of the greatest importance in disease transmission. We are all very careless in our habits in regard to saliva, far

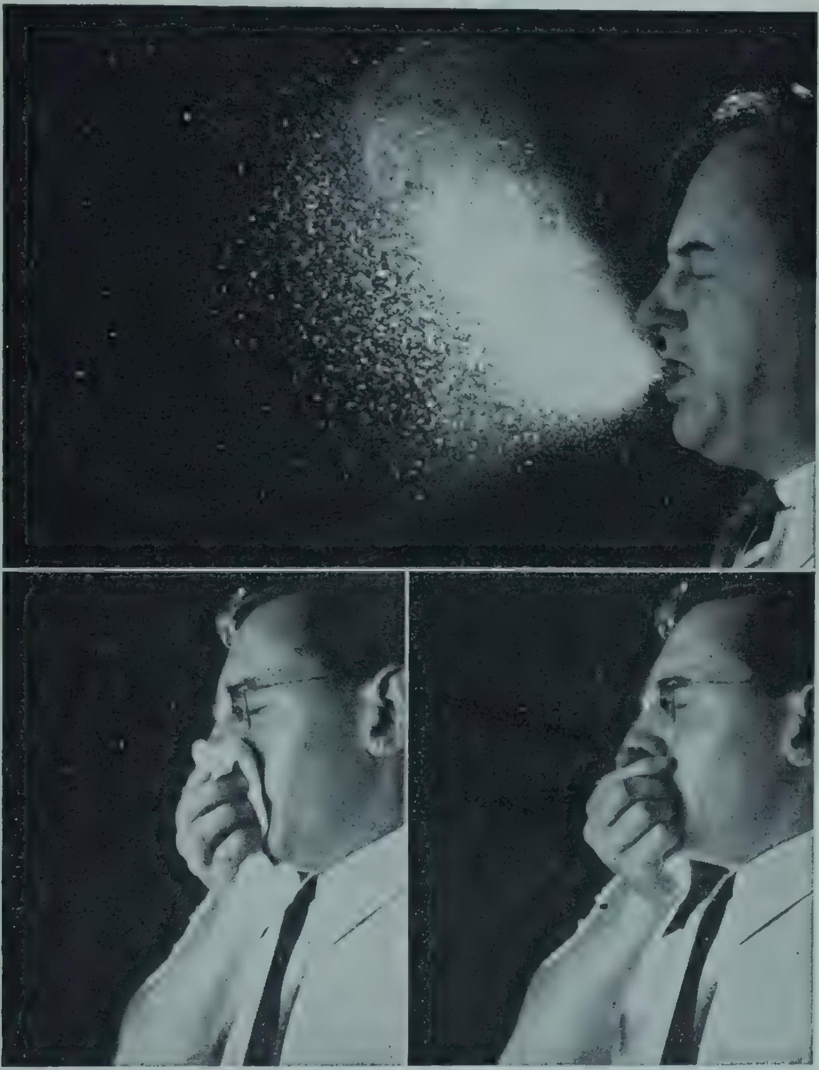

Fig. 155. *Upper,* Unstifled sneeze explodes a cloud of highly atomized baceria-laden droplets. Some droplets travel at such high speed that they are streaks even at 1/30,000 of a second. *Lower left,* Stifled by handkerchief, only few sneeze droplets escape to infect the atmosphere. *Lower right,* Stifling by hand, although less effective than handkerchief, stops most droplets. (Courtesy of M. W. Jennison, Depart. of Plant Sciences, Syracuse University.)

more so than we like to realize. The case has been stated vividly by a famous physician: "If infection by contact is of such very great importance in the fecal-borne diseases, how much more important must it be in diseases in which the infective agent is found in the secretions of the nose and mouth, as is the case with diphtheria, scarlet fever, smallpox, mumps, measles, whooping cough, tuberculosis, influenza, and cerebrospinal meningitis. Every one avoids feces and urine, but it is only the very few who have any objection to saliva.

"Not only is the saliva made use of for a great variety of purposes, and numberless articles are for one reason or another placed in the mouth, but for no reason whatever, and all unconsciously, the fingers are with great frequency raised to the lips or to the nose. Who can doubt that if the salivary glands secreted indigo the fingers would continually be stained a deep blue."

Droplets of saliva are presumably responsible for much disease transmission. Every cough or sneeze results in a germ-laden spray. Sneezing or coughing in public without a handkerchief is reprehensible but commonplace (Fig. 155). The droplets may remain suspended for some time in the air and may be carried many feet by draughts. The droplets may become dry; the mucous-coated bacteria, which then constitute what are called *droplet nuclei,* float about through the air like dust particles.

The possibilities of disinfecting air in public meeting places, operating rooms, etc., have been the subject of intensive and large scale investigation. The two methods giving most promise are irradiation with ultraviolet light and the use of bactericidal vapors, sometimes called aerosols. These are discussed in more detail in the chapter on bacteria in the air.[12]

Transmission by Dust. Little imagination is needed to understand how disease may be transmitted by dust. Particles of saliva or sputum containing bacteria fall to the floor, bedding or clothing, dry quickly and, if not exposed to excessive heat or sunlight or other unfavorable influences, the bacteria in them may survive for considerable periods. When the dust is stirred up, persons inhaling it or getting it into operative or accidental wounds may suffer an attack of disease. Probably the respiratory diseases like tuberculosis, pneumonia, diphtheria and scarlet fever are often transmitted by such means, since the organisms involved resist drying and are spread in dust. A good example of a dust-borne disease is coccidioidomycosis (see page 405). The dust in places where psittacine birds (parrots, etc.) are raised and sold was formerly a source of much infection with the virus of "parrot fever" (psittacosis), since the virus occurs in feces and nasal secretions of infected birds.

In barracks and hospital wards dust and lint from clothing and bedding are important means of disease transmission, especially of respiratory infections. One method of controlling this is to impregnate bedding with imperceptible oils which tend to keep the dust from flying about. Floors and sweepings are also oiled. The oil merely controls dust. It does not kill microorganisms.[13]

Transmission by Foods (Other than Milk). These problems are discussed in Chapter 38.

Transmission by Direct Contact. This means of disease transmission needs little comment. Obviously, if one rubs against infectious material he runs a risk of infection. Usually it is easy to guard against such an eventuality, especially if one avoids transferring the *contagium* to its special portal of entry. One does not voluntarily come into physical contact with feces, sputum or the visible sores or pustules of infected persons. However, an innocent kiss may transmit tuberculosis, pneumonia, scarlet fever, diphtheria and other diseases and doubtless does with tragic results, especially to infants and young children and very old persons. The best examples of contact-transmitted diseases of adults are syphilis and gonorrhea, both spread by coitus, the former by kissing also.

BIOLOGICAL TRANSMISSION OF DISEASE

Biting Insects. Many bacteria are associated with insects.[14, 15, 16] There are several diseases the sole natural means of transmission of which is the bite of insects. The classical observations of Smith and Kilborne in 1893 on transmission of Texas fever of cattle by the cattle tick (*Boophilus annulatus*) were the first report on tick transmission of disease, although certain mosquitoes and other insects had previously been found to transmit other parasites (malaria, filaria worms, etc.). Usually, but not always, each disease has its own specific insect vector. Malaria is the best known disease of this type, being carried only by certain mosquitoes (*Anopheles*). Dengue fever, yellow fever, African sleeping sickness, typhus (not typhoid), Rocky Mountain spotted fever, tularemia, and some other diseases, are also transmitted by the bites of certain specific insects (mosquitoes, ticks, lice, fleas). Plague is an outstanding example of a disease transmitted by biting insects (rat fleas) and has played an important role in the history of nations. Tick-borne diseases, like Rocky Mountain spotted fever and possibly tularemia, transmitted among rabbits and from them to man, may be carried by birds, as rabbit ticks have been found making long journeys attached to birds.

During World War II insects were an important source of disease as shown by the following data:*

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* From the bulletin of the U.S. Army Medical Department, 1946, *5*:613.

Disease*	Insect	Cases reported
Malaria	Mosquitoes	440,789
Dengue	Mosquitoes	82,392
Sandfly fever	Phlebotomus fly	12,228
Typhus (not typhoid)	Lice, ticks, mites	7,269
Filariasis	Mosquitoes	2,110
Leishmaniasis	Phlebotomus fly (?)	344
Relapsing fever	Ticks, lice	241
Rocky Mountain spotted fever	Ticks	60

Insect Feces. Cockroaches were shown by Chambers as early as 1914[17] to transfer cholera vibrios in their intestines for at least forty-eight hours after feeding on human cholera feces. Ants transmit the disease, and possibly others, in the same manner. Flies have long been under indictment for the same crimes.[18]

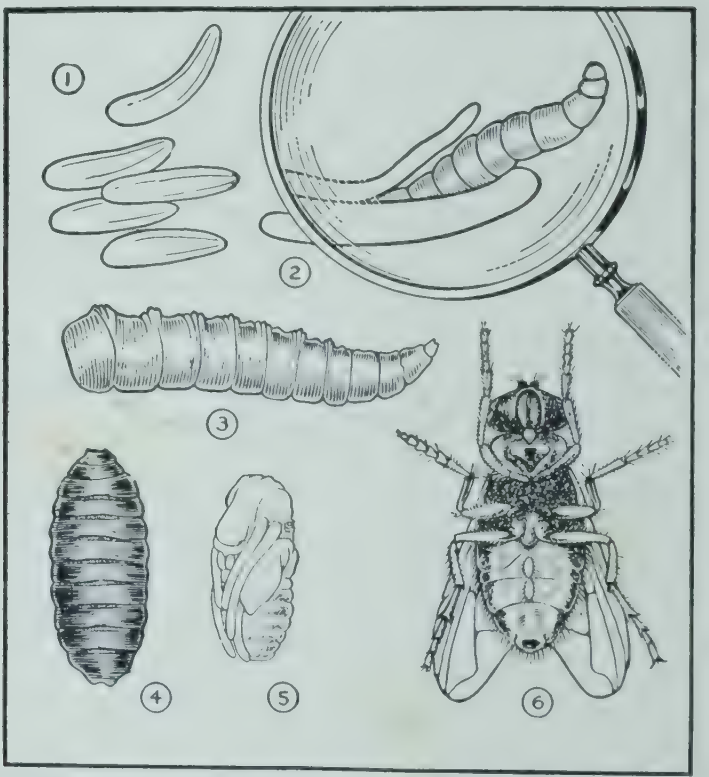

Fig. 156. Life history of the house fly. 1, Eggs of the housefly. 2, Larva or maggot hatching from egg. 3, Larva. 4, Pupa in case. 5, Nearly developed pupa. 6, Mature house fly as seen when walking up the outside of a window pane. (Community Health, by Turner, C. E., and Collins, G. B., published by D. C. Heath.)

The feces of lice infected with typhus organisms will infect if scratched into the skin. Feces of infected fleas contain plague bacilli and may contaminate small wounds or scratches. Indeed, many blood-sucking insects may pass infective agents in the feces and may also cause infection by being crushed on the skin near, or in, an abrasion or wound. Engorged ticks are especially dangerous in this respect because they contain a relatively large volume of blood. Flies may also transmit infantile paralysis by fecal contamination, as the virus has been demonstrated to occur in flies.[19, 20]

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* Some of these are described elsewhere in this book (see index).

Bodies of Insects. *Insects which fly* from unsanitary, unscreened and undisinfected privies or hospitals to dwellings may mechanically transmit intestinal and other disease organisms on their feet and bodies. In areas where flies abound, especially rural or city slum areas, if there is access to infectious sewage or feces, enteric fevers are likely to be more prevalent especially during the summer months when flies are numerous. In places where city sewerage systems are not available, fly-borne disease can be avoided to a large extent by the construction of screened and deep-pit or other sanitary types of privies or, better still, by the installation of sanitary plumbing and septic tanks. Plans and specifications for such structures can be obtained from State Health Departments.

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Section II

YEASTS, MOLDS AND HIGHER BACTERIA

Introduction

In Section I of this book the student has been introduced to the general field of microbiology, including the methods used in its study and various physical and chemical factors affecting microorganisms, classification schemes, metabolism, and other information basic to an understanding of the whole subject. The remainder of the book will deal with microorganisms themselves.

The first two chapters of Section II will afford a general view of two large groups of microorganisms, important in medicine, agriculture and industry, closely related to bacteria, namely, the yeasts and the molds. These serve well for introductory studies because they are easily seen, are conveniently manipulated and cultivated in much the same manner as bacteria and because they illustrate several phenomena of fundamental significance in microbiology. Following the discussion of yeasts and molds several chapters are devoted to still more primitive organisms called "higher bacteria" because they are somewhat more complex than true bacteria. The simplest or true bacteria, viruses, etc., are then discussed in subsequent parts of the book.

Chapter 21

THE YEASTS

Although neither yeasts nor molds are included in the class Schizomycetes, they belong to the same major division of the vegetable kingdom, namely, the Mycophyta or fungi. They resemble bacteria in many respects and are sometimes very annoying contaminants in the laboratory, preserving kitchen or industrial process.

The general relationships of the yeasts and molds to the other fungi are seen in the table on page 376 and that given below.

Relationships of the Yeasts, Molds and Bacteria

FUNGI
1. Lichenes (Lichens)
2. Basidiomycetes (Mushrooms, puff-balls, bracket fungi, etc.)

Yeasts and molds:
3. Phycomycetes (Not sac-forming; filaments not septate)
4. Ascomycetes (Sac-forming; filaments septate)
    - I. Yeast-like
    - II. Mold-like
5. Fungi imperfecti (Mainly like Ascomycetes II, but no sexual phenomena seen)

6. Schizomycetes (Bacteria)

Further details of the relationships of yeasts and molds are seen on pages 374, 375, 412.

Many common species are of great importance in industry, both as the cause of fermentations yielding valuable substances like isopropyl alcohol, acetone, etc., and because of the damage they cause through decay, "mildew," etc. Many are the cause of diseases of plants, animals and man. Several molds, such as *Penicillium* and *Aspergillus,* have assumed enormous importance as the sources of agents exceedingly useful in controlling infections due to bacteria (see section on bacteriotoxic substances, page 239).

Molds and yeasts are often encountered by the bacteriologist incidentally. Also, in descriptions of bacteria, comparisons are often made with yeasts or molds and historical and other references are made to them.

Like most bacteria, and all other fungi, yeasts and molds are devoid of photosynthetic pigments and they grow best in dark or shady places. While, in general, the individual cellular elements composing them are relatively enormous as compared with bacteria, they are nevertheless so minute as to require the use of the microscope for their proper investigation. Yeasts and molds, especially when once isolated on artificial media, may be cultivated on much the same sort of materials as are used for bacteria and are stained and manipulated in much the same way. Certain media are especially recommended for the cultivation of these organisms, a good illustration of the general type being that of Sabouraud.* Most of the media for molds and yeasts contain considerable amounts of carbohydrate. The reaction of this type of medium is slightly acid (*p*H about 5.5). Slight acidity seems to favor the growth of many molds and yeasts and inhibits the growth of some kinds of contaminating bacteria.

A synthetic medium much used for studying molds and yeasts is that of Czapek-Dox-Thom.† Media may also be made from water in which cereals, vegetables, or meat have been soaked or cooked. Carbohydrate and agar are added, the *p*H adjusted to around 5.5, and the material is sterilized. For more complete details the student should consult the literature.[1] In general, the yeasts and molds have nutrient requirements similar to those of the saprophytic bacteria except that they require more carbohydrate as a rule.

The yeasts and molds are, as a rule, biochemically versatile and active, metabolizing or attacking a great variety of chemical compounds, and in the soil accomplish much of the decay of organic matter so necessary to life and fertility of the land.

Structurally and physiologically the yeasts and molds are more complex than the bacteria, and may, therefore, be regarded as more highly evolved plants.

Classification of Yeasts and Molds. The differentiation between yeasts and molds is often difficult. Indeed, the transition in form, and manner of reproduction, from one group to another is so gradual

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* *Sabouraud's agar:*

Water	1000 ml.
Peptone	10 gm.
Dextrose (or Maltose)	20 gm.
Agar	20 gm.

Dissolve all ingredients in water. Sterilize by autoclaving. The *p*H should be about 5.0–5.6.

† *Czapek's solution (modified by Dox and Thom):*

Sucrose	30.0 gm.
$NaNO_3$	2.0 gm.
K_2HPO_4	1.0 gm.
$MgSO_4$	0.5 gm.
KCl	0.5 gm.
$FeSO_4$	0.01 gm.
H_2O	1000.0 ml.

Agar may be added to 1.5 per cent concentration. The reaction should be around *p*H 6.8.

that it is difficult to draw sharp lines of demarcation, and therefore existing classifications are, like classifications of bacteria, somewhat artificial and arbitrary.

For present purposes it is sufficient to indicate the main divisions of the group of fungi, leaving finer systematization for more advanced students of the subject. The following table, adapted from a number of different sources, while incomplete is a satisfactory working basis for the present discussion:

GENERAL RELATIONSHIPS OF YEASTS AND MOLDS

1. *Phycomycetes* (not sac-forming; filaments not septate)

Asexual spores enclosed in *sporangia*. Sexual spores are free (*zygospores*).	Aquatic forms cause diseases on fish; some are found in aquatic plants. Example *Saprolegnia*. Common terrestrial forms are *Mucor mucedo* ("manure mold," white) and *Rhizopus nigricans* ("bread mold," black).

2. *Ascomycetes* (sac-forming; filaments septate)

Group I (*yeast-like*)	
Not characteristically filamentous. Cells oval or elliptical. Sexual or asexual spores (when present) enclosed in sacs called *asci*.	*Saccharomyces* true *Schizosaccharomyces* yeasts *Oidium* (short filaments). Cup fungi, powdery mildews, morels.
Group II (*mold-like*)	
Characteristically filamentous. *Septate*. Asexual conidia* borne openly on tips of fertile hyphae. Sexual spores borne in sacs called *perithecia*.	*Aspergillus glaucus* (green mold, common on bread) *Penicillium citrinum* (blue mold, common on decayed lemons).

3. *Fungi imperfecti*

Not assignable to any of the preceding groups. Most fungi imperfecti might belong in group II (the filamentous species of the Ascomycetes) but *no sexual spores or perithecia* have been demonstrated. Torulopsis or Torula (non-spore forming yeast-like fungi) might be assigned to this group.	Many fungi causing potato and bean diseases belong here. Many ringworm fungi and those causing "athlete's foot" also are placed here pending more complete studies.

There are many technical terms in this classification wholly unfamiliar to one who has so far encountered only bacteria in his study of microorganisms. However, these terms will become familiar after a more detailed discussion of the various groups, during which the outline serves as a reference point and a guide. A convenient diagram of the relationships of the yeasts and molds to each other and other fungi is shown below.

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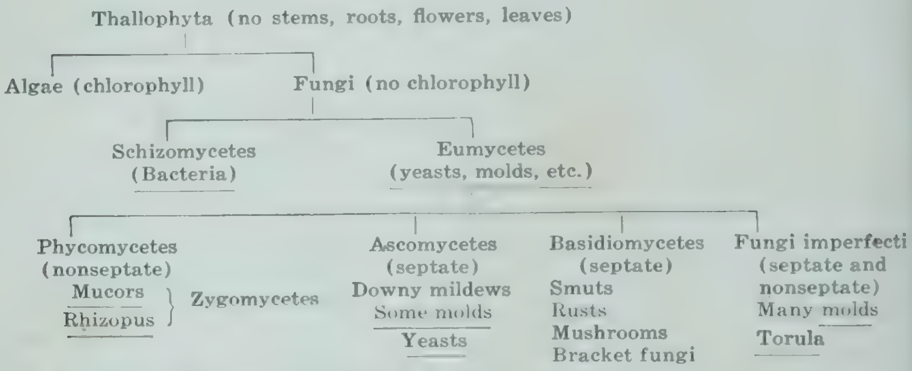
* Conidium is derived from a Greek word meaning dust.

Classification of Fungi


Thallophyta (no stems, roots, flowers, leaves)
Algae (chlorophyll)
Fungi (no chlorophyll)
Schizomycetes
(Bacteria)
Eumycetes
(yeasts, molds, etc.)
Phycomycetes
(nonseptate)
Mucors
Rhizopus
Zygomycetes
Ascomycetes
(septate)
Downy mildews
Some molds
Yeasts
Basidiomycetes
(septate)
Smuts
Rusts
Mushrooms
Bracket fungi
Fungi imperfecti
(septate and
nonseptate)
Many molds
Torula

(Only those underlined are considered in detail in this book.)

THE YEASTS

Because they are structurally among the simplest of the true fungi, the yeasts will be discussed first.

The term *yeast* cannot be accurately defined. A person slightly familiar with the group might think of yeasts as unicellular fungi, never forming *mycelia** (the woolly molds as commonly seen), containing small but definite nuclei, reproducing by budding and ascospore formation and often sexually by zygote† formation or otherwise. The chief difficulty with this, as with most definitions of biological phenomena, is that there are many "exceptions." For example, some yeasts form more or less definite mycelia under certain conditions. On the other hand, many filamentous molds form yeast-like cells, especially under conditions of reduced oxygen tension. Some supposed yeasts are merely unicellular phases of higher molds, including the Basidiomycetes. For convenience, however, we may use the crude definition given above as a basis for discussion, bearing in mind that the group as a whole is not clearly differentiated but is linked by many slightly differing transitional forms with other large groups of filamentous fungi. The yeasts may be regarded as Ascomycetes which have wholly or partly lost the power of filament formation.

Structure of Yeast Cells. The cells of yeasts are quite different, in many respects, from bacterial cells. Morphologically, they are oval or ellipsoidal, rarely forming very long filaments. Yeast cells are, on the average, much larger than bacterial cells. Some oval yeast cells may have a volume thousands of times that of *Staphylococcus* cells and a long diameter up to 30 microns or more.

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* A mycelium is a mass of woolly, filamentous mold growth. The individual filaments are called hyphae.

† A zygote is a cell formed by the union of two gametes or reproductive cells. In yeasts there is no apparent differentiation of sex cells or gametes, but conjugation between like cells occurs.

Yeast cells possess all the principal structures found in cells of metaphytes. There is a well-differentiated but small nucleus and a thick cell wall, probably composed of a cellulose-like substance.

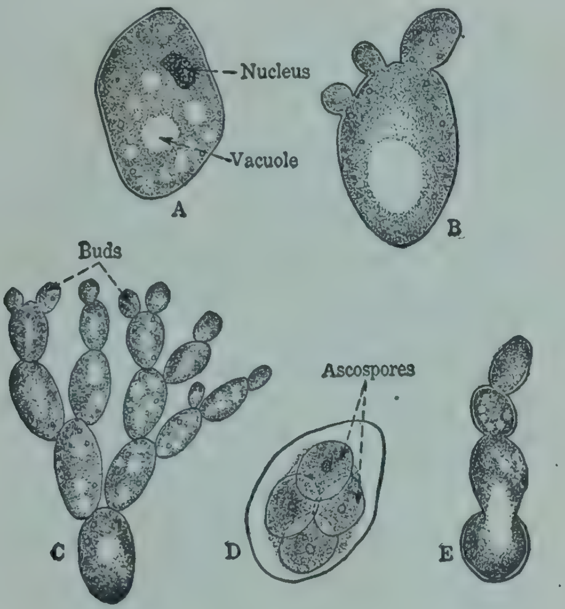

Fig. 157. Yeast (*Saccharomyces cerevisiae*). *A*, Single cell highly magnified; *B*, cell in process of budding; *C*, chain of cells formed as result of rapid budding and growth; *D*, formation of ascospores; *E*, germination of ascospore and the development of new plants by budding. (Redrawn from Curtis. Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," John Wiley & Sons, Inc., publishers.)

There are definite chromosomes and heredity mechanisms following Mendelian laws.[2] There are also numerous vacuoles containing food or waste substances and there are granules of various kinds, some evidently related to the formation and storage of glycogen, others composed of volutin. Yeasts sometimes contain large quantities of fat, of which commercial use may be made.

On the other hand, yeasts resemble many bacteria in being unicellular, nonmotile, devoid of chlorophyll, and plant-like in requiring food material which is wholly soluble and can pass through the cell wall by diffusion. They may be cultivated on ordinary bacteriological media, as well as on such special media as have been mentioned, stained by the same methods (they are gram-positive) and, in general, manipulated in the same way. Yeasts also undergo rough ⇆ smooth and other variations much as do bacteria, and the phenomena are probably closely related. Encapsulated yeasts have been described.[3] In some, these capsules consist of starch.[4]

Multiplication of Yeasts. Yeasts may multiply by one or more of four methods. First, they multiply by a method called "budding" in which large, mature cells divide, each giving rise to one or more daughter cells which are at first much smaller and which may cling to the parent cell (Fig. 157), often even after the daughter cell has divided. Clumps and chains of cells are thus formed.

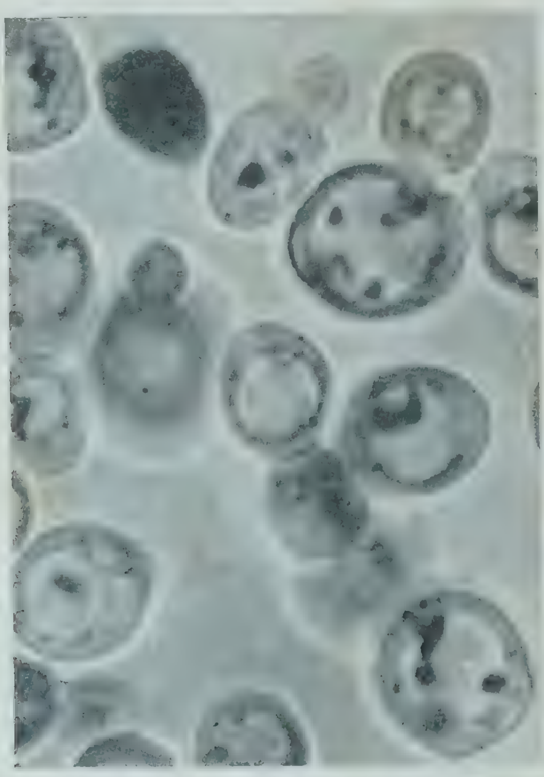

Fig. 158. Yeast cells showing budding and nuclear structures, vacuoles, etc. (Lindegren, C. C., in Bact. Reviews, vol. 9.)

Second, some species of yeast, in the genus *Schizosaccharomyces*, divide by equal (binary) fission, much as do the bacteria.

Third, *ascopores* (spores within a sac or *ascus*) are formed within a single cell when the nucleus undergoes 1, 2 or 3 divisions, without participation of the rest of the cell, forming 2, 4 or 8 ascospores in the sac. There is no apparent involvement of sexes in this process (see Fig. 157). Yeasts are classed in the group of Ascomycetes or sac-forming fungi because of these spores enclosed in an ascus (Figs. 158, 159, 160).

The number of ascospores per cell tends to be characteristic of the species. These ascospores are, in many respects, analogous to bacterial spores, being resistant to climatic heat, drought, and other unfavorable environmental conditions. They are not so thermo-resistant as bacterial spores, however, being killed by 60° C. in a short time. Since they are generally produced in groups of two or more they represent a process of multiplication as well as preservation, thus differing from bacterial spores, of which only one is produced by each individual.

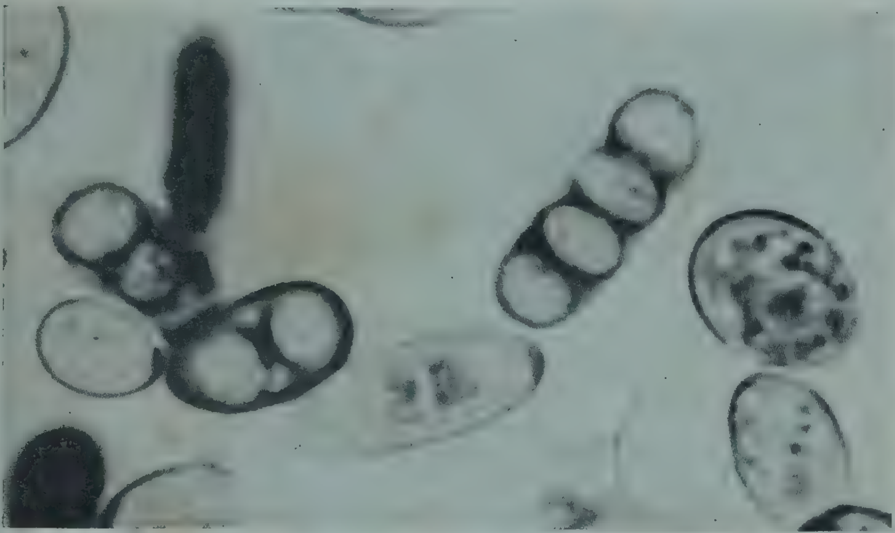

Fig. 159. *Saccharomyces cerevisiae* showing asci with two and four ascospores, as well as vegetative cells. (Lindegren, C. C., in Bact. Reviews, vol. 9.)

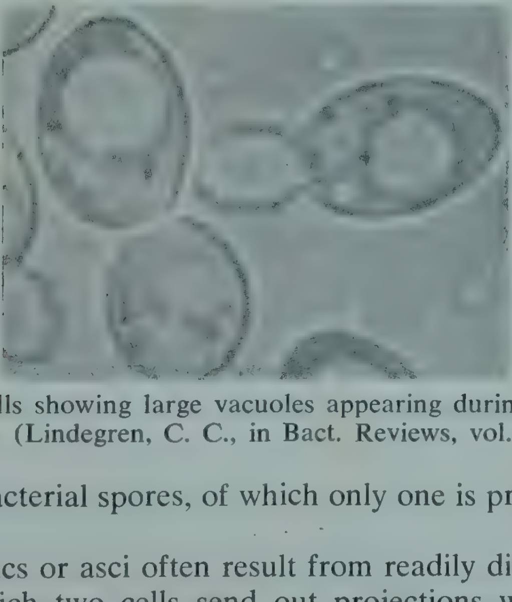

Fig. 160. Yeast cells showing large vacuoles appearing during process of budding. (Lindegren, C. C., in Bact. Reviews, vol. 9.)

Fourth, the sacs or asci often result from readily discernible sexual processes in which two cells send out projections which meet and form a copulation canal and undergo nuclear intermingling, etc.; the whole process is somewhat suggestive of that seen in algae like *Spirogyra* or in certain of the filamentous molds. The resulting cell resembles a zygospore.* The nucleus of this cell divides to form a number of *ascospores* (Fig. 161).

One interesting point about the reproductive processes of yeast is the probability that in both budding and ascospore formation the nucleus undergoes mitotic division. Haploid† and diploid† cells are produced by some species. By placing haploid spores of different species together under favorable circumstances conjugation will occur with the formation of a diploid zygote which germinates into a hybrid yeast. This hybridization of yeasts offers many possibilities to the student of mycology and genetics, especially for commercial purposes.[1] The bacteriologist is forced to content himself with contemplation of mere fission.

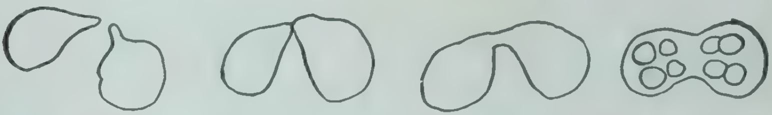

Fig. 161. Composite diagram of the process of copulation in a species of yeast. Note the ascospores in the fertilized cells. (Redrawn from Guilliermond.)

Chlamydospores. Many yeasts and molds, and probably bacteria, are capable of forming these bodies. Active growth of the cell ceases, food is stored, the cell acquires a thick protective wall, dehydration takes place, and the resulting dormant cell tides the plant over unfavorable conditions. It is called a *chlamydospore*. It is possible that similar cells are produced by some bacteria, streptococci, for example. Such cells may be seen in yeast cultures or strung like beads along the mycelia of molds such as *Mucor*. No increase in nuclei occurs as in ascospore formation and the chlamydospore is therefore not reproductive in function. Such bodies are not as heat resistant as bacterial spores.

Habitat of Yeasts. Yeasts are widely distributed in nature. They commonly occur on grapes and other fruits, vegetables, etc., and multiply in the juices of the fruits, etc., which they decompose. The spores pass the winter in the soil. The kind of wine made from grapes depends to some extent on the varieties of yeasts occurring upon them naturally. Yeasts and torulae (asporogenous,‡ yeastlike fungi) may also be found in dung, soil, water and milk, and are not infrequently observed in cultures made with swabbings from the throat. They are found in insects, flowers, honey, etc., etc.

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* A zygospore is a cell, dormant in character, produced by two similar gametes. These are commonly produced by certain algae and molds (see page 393)

† Haploid means nuclei with half the full number of chromosomes; diploid cells have the full complement of chromosomes.

‡ Nonsporeforming.

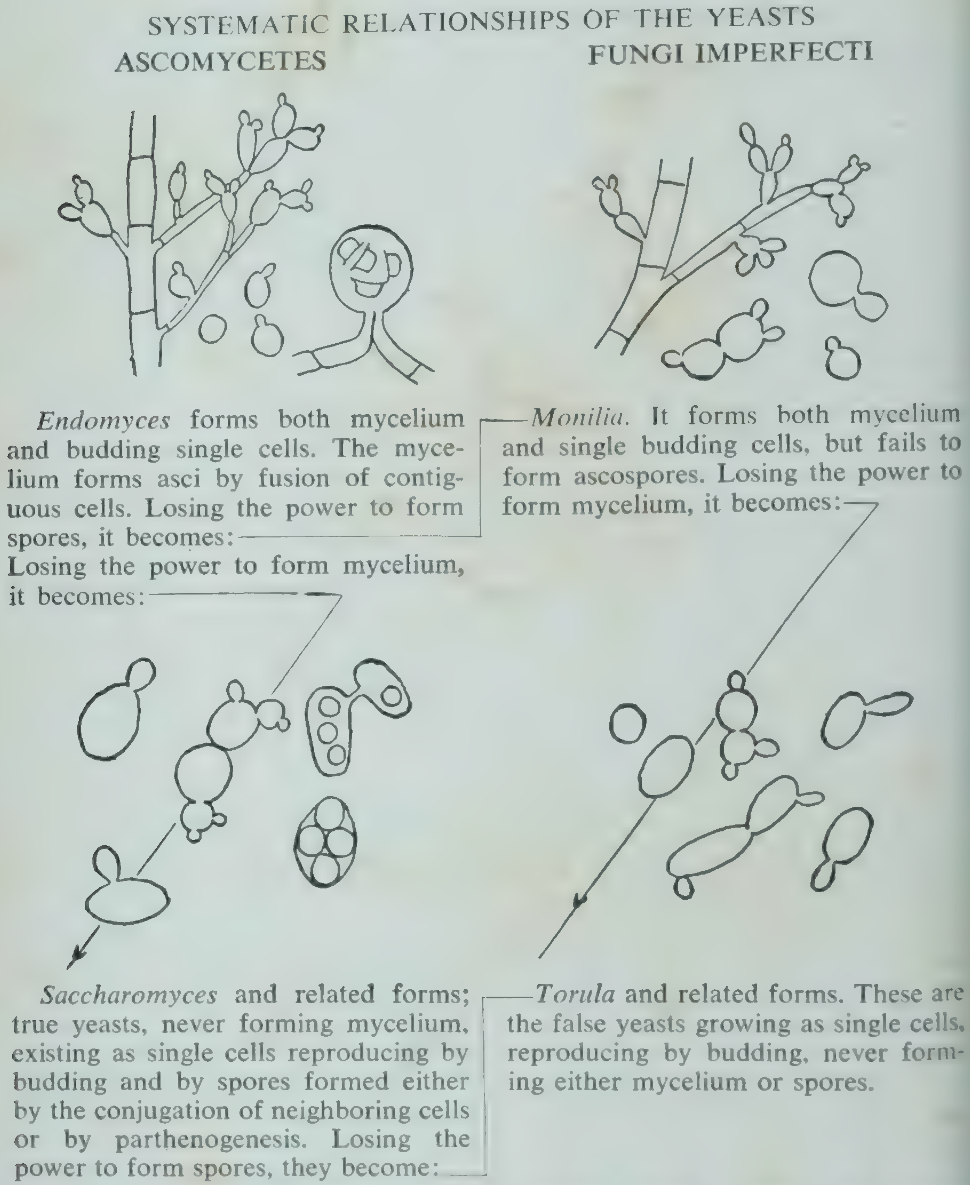
Activities of Yeasts. Yeasts, especially the *Saccharomyces,* are characteristically fermentative organisms, producing chiefly alcohol and carbon dioxide from sugar under anaerobic conditions of growth. Their alcohol-forming power is used in the manufacture of wines and beer and also in the commercial production of alcohol. Varieties especially adapted to each purpose are used. Thus, there are "distillery yeasts," "top yeasts," for beer, and "bottom yeasts" for beer and wine. Distillery yeasts are the better alcohol producers. The yeasts grow in the beer-wort or fruit juice, utilizing the nutrient substances there. Sterilized beer-wort agar is commonly used for the cultivation of yeasts.

The carbon dioxide-producing power of yeasts is important in baking. Sugar and milk are mixed in the dough. The yeast, utilizing the nutrient substances of the dough for cell synthesis, metabolizes the sugar as a source of energy and bubbles of carbon dioxide are formed which "raise" the bread. Alcohol is also formed. Baking bursts the starch granules, coagulates and dries the matrix so that the bread retains its form, and drives off the alcohol. Some yeasts synthesize many vitamins, including riboflavin.[5] Others require a number of vitamins and this makes them useful in the assay of vitamins by fermentation methods (see page 696). The cell physiology of yeasts and molds, except as related to reproduction, is basically much like that of bacteria.

Classification of Yeasts. A generally satisfactory classification of yeasts is difficult to prepare. Some taxonomists would include with yeast sporogenous organisms which consist entirely or largely of mycelial growth or regularly produce definite mycelia; others would exclude these and class as yeasts only those which never, or rarely, produce mycelium, and then very little.[6] In the present discussion organisms producing extensive mycelia, although mentioned in the table, are not discussed in detail with the yeasts. The possible evolutionary relationships of four types of yeasts and yeastlike organisms are seen in Figure 162. The organisms shown in Figure 162 are discussed farther on.

A very useful new method of classifying yeasts on the basis of their ability to utilize various carbon compounds (sugars, alcohols, etc.) has been worked out by Wickerham and Burton. The basal medium is a simple synthetic one and the carbon source is the determining factor in growth. The method is based on *ability to attack* the carbon source regardless of the products of metabolism.[5a]

We may, for convenience in the present discussion, divide the yeast-like organisms into two great groups. Endomycetaceae and Torulaceae, as shown in the table on page 383. The first group includes ascospore-formers; the second group includes species of yeastlike organisms



SYSTEMATIC RELATIONSHIPS OF THE YEASTS

ASCOMYCETES

FUNGI IMPERFECTI

Endomyces forms both mycelium and budding single cells. The mycelium forms asci by fusion of contiguous cells. Losing the power to form spores, it becomes:
Losing the power to form mycelium, it becomes:

Monilia. It forms both mycelium and single budding cells, but fails to form ascospores. Losing the power to form mycelium, it becomes:

Saccharomyces and related forms; true yeasts, never forming mycelium, existing as single cells reproducing by budding and by spores formed either by the conjugation of neighboring cells or by parthenogenesis. Losing the power to form spores, they become:

Torula and related forms. These are the false yeasts growing as single cells, reproducing by budding, never forming either mycelium or spores.

Fig. 162. (Reprinted by permission from Henrici, "Molds, Yeasts, and Actinomycetes," John Wiley & Sons, Inc., 2nd ed., 1947.)

which do not form ascospores. As pointed out above, the mycelium-formers of the first group are named in the table but are not further discussed in the text of this chapter.

CLASSIFICATION OF YEASTS

- I. ENDOMYCETACEAE (Ascospores formed).
  - 1. Principally (or largely) mycelial growth.
    - (1) Eremascoideae
      - (Eremascus)
    - (2) Saccharomycoideae
  - 2. Mycelia with oidia, or oidia alone.
    - (1) Endomycoideae
      - (Endomyces)
    - (2) Endomycopseae
      - (Endomycopsis)
    - (3) Nematosporoideae (mycelia and budding yeast cells; ascospores—spindle-shaped)
      - (Monosporella)
      - (Nematospora)
      - (Coccidiascus)

Forms intermediate between molds and true yeasts.

(Not included in text discussion.)

- 3. No mycelia. (Multipolar budding.)
  - (1) Saccharomyceteae
    - (*a*) *Saccharomyces*
    - (*b*) *Torulaspora*
    - (*c*) *Pichia*
    - (*d*) *Willia*
    - (*e*) *Debaromyces*
    - (*f*) *Schwanniomyces*
    - (*g*) *Schizosaccharomyces*
  - (2) Nadsonieae
    - (*a*) *Saccharomycodes*
    - (*b*) *Hanseniaspora*
    - (*c*) *Nadsonia*

Yeasts of commercial importance; some as nuisances, some as valuable aids, to bakers, brewers, distillers, commercial alcohol production, wine makers, etc. Some species also common in soil, dust, etc.

- II. TORULACEAE OR ANASCOSPOROGENOUS YEASTS (ascospores not formed). Belong to the *Fungi imperfecti;* often called "wild yeasts."
  - 1. With conidia.
    - (1) Nectaromycetaceae (occur in the nectar of flowers)
  - 2. Without conidia.
    - (1) Torulopsidaceae (no pigments)
      - (*A*) *Torulopsidoideae* (no pseudomycelium)
        - *Torulopsis* (Some pathogens; mostly saprophytes of soil, dust, dung, etc.)
        - *Pityrosporum* (Possibly related to diseases of scalp)
        - *Asporomyces*
        - *Klockera*
        - *Trigonopsis*
        - *Schizoblastosporion* (various skin lesions)
        - *Mycoderma* (common in dust and vinegar)
      - (*B*) *Mycotoruloideae* (*Pseudomycelium formed*)
        - *Candida* (Various pathogens causing dermatitis, etc.)
        - *Trichosporon* (Skin diseases)
    - (2) Rhodotorulaceae (carotenoid pigments)
      - Various pink, red, or salmon-colored varieties, as *Rhodotorula* (Often cause spoilage and discoloration of foods)

I. ENDOMYCETACEAE. The sacformers, or ascosporogenous yeasts, include the tribes (1) Saccharomyceteae and (2) Nadsonieae, in both of which mycelium formation is reduced to a minimum.

1. Saccharomyceteae. This tribe includes the genus *Saccharomyces*, which is the largest and most familiar genus and in which are found most of the common yeasts of commerce, such as *S. cerevisiae* and *S. ellipsoideus*. Other genera in the tribe are *Torulaspora, Pichia, Willia, Debaromyces, Schwanniomyces* and *Schizosaccharomyces*. All of these produce buds at various parts of the parent cell, *i.e.*, budding is *not bipolar*. The various genera may be differentiated from one another by fermentation and other biochemical methods and by the shapes of their ascospores or by their modes of germination or modes of formation of ascospores (sexual or asexual). Some are found in beer, others in wine vats, dung, soil, etc.

The *Saccharomyces* are the most common and valuable industrial yeasts. Sexual phenomena are found in this genus,[1] budding and ascospore formation being additional means of multiplication. The cells are oval or elliptical, and oval buds are produced at any part of the cell surfaces (Fig. 163, 8). As mentioned above, their fermentative powers are very useful. They are the servants of the baker and brewer. There are numerous species having various special properties of use in industrial processes such as brewing, power-alcohol manufacture, etc.

Torulaspora cells form ascospores by simply rounding up and developing a conspicuous fat globule in the center of the cell (Fig. 163, 3). The ascospore in this species consists really of the modified whole cell. Conjugation does not occur frequently, if at all, although tubes like copulation tubes are formed.

Pichia and *Willia* (Fig. 163, 1) are similar genera. Their chief industrial importance is as nuisances in the wine industry since they attack sugar in fruit juices without producing alcohol, and metabolize alcohol in fermenting wine. They give off a very characteristic odor of ethyl acetate and related esters, suggestive of piles of bruised apples fermenting in the summer sun. Their growth in wine vats is in the form of a scum on the surface. *Pichia* is recognizable by its elongated cells, strung together in a distinctive manner, and by its hemispherical or angular spores containing distinct fat globules. *Willia* produces either spores which are hemispherical, having a projecting annular fin like the brim of a derby hat (*W. anomala*) or spores which are flatter with an equatorial projecting fin suggestive of the ring of Saturn (*W. saturna*). The fat globule is distinct.

Debaromyces (Fig. 163, 4) form ascospores after conjugation, a single spore to a cell. This species occurs in soil, dust, etc.

The *Schwanniomyces* (Fig. 163, 7) are interesting in that, like *Torulaspora,* under conditions which support spore formation the cells send out elongated processes suggestive of copulation canals, but fusion rarely, if ever occurs. A single ascospore then develops in each cell, the entire cell constituting a spore, as in *Torulaspora* and *Nadsonia.*

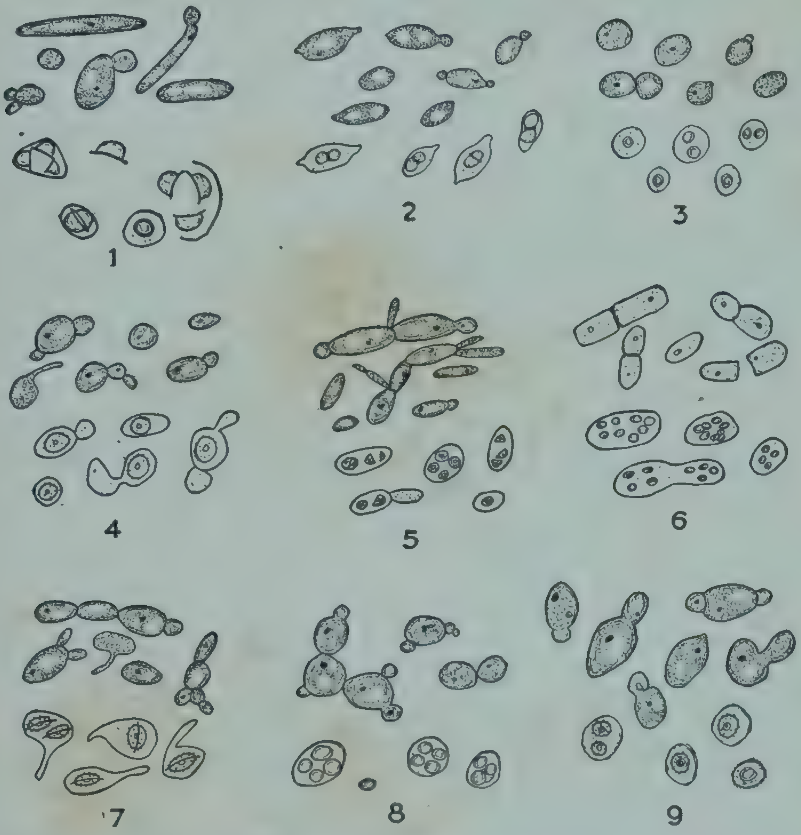


Fig. 163. Various forms of yeasts, showing vegetative and budding cells and ascospores. 1. *Willia;* 2. *Hanseniaspora;* 3. *Torulaspora;* 4. *Debaromyces;* 5. *Pichia;* 6. *Schizosaccharomyces;* 7. *Schwanniomyces;* 8. *Saccharomyces;* 9. *Nadsonia.*

The *Schizosaccharomyces* (Fig. 163, 6) differ from all other yeasts in reproducing by binary fission like bacteria. They also reproduce by conjugation of seemingly identical cells; eight ascospores result after division of the fused nuclei. Sometimes fusion occurs between a cell and its bud, somewhat as in *Nadsonia.* This process is called heterogamy or heterogamic copulation.

2. Nadsonieae. The cells of yeasts of the tribe Nadsonieae are characterized by *bipolar budding.* There are three genera: *Saccharomycodes, Hanseniaspora* and *Nadsonia.*

Saccharomycodes are not very common, occurring in decaying organic matter and in soil. The daughter cell is characteristically separated from the parent cell by the formation of a septum between the two, which splits, freeing the daughter cell. The cells are very large.

Hanseniaspora are generally easily recognizable by their lemon-shaped cells and rounded spores with a ringlike projection. One or two spores may be formed per ascus. These yeasts are of relatively little industrial importance although they occur in wines and on fruits (Fig. 163, 2).

Nadsonia cells (Fig. 163, 9) are large and oval, often showing a small protruberance at one end or buds at both ends. After one of the daughter cells separates, the nucleus of the other daughter fuses with that of the parent cell. The combined cells fuse to form one large spore of irregular form and containing a large fat globule. The growth of some species, as *N. fulvescens,* is sometimes very mucoid and the fat in older cultures may give a yellow or brownish color to the growth.

II. TORULACEAE OR NON-SPOREFORMING YEASTS. There are several subdivisions of yeastlike plants forming no ascospores. A small group, rather specialized as to form and habitat, the Nectaromycetaceae occur mainly in honey and insects. The largest group, containing several genera, is called Torulopsidaceae. These organisms closely resemble the yeasts in most respects except that they have not been observed to produce ascospores by any method. They are, therefore, part of the heterogeneous group known as Fungi imperfecti. Their cells are usually more nearly spherical than are the cells of yeasts. Their classification is not complete. The largest and commonest genus is *Torulopsis,* commonly called torula. Various kinds of torula have been found causing "diseases" of beer and other fermented foods. They are common in soil, water and dust. Some few species are pathogenic, infecting the brain and meninges.

Another interesting group, called Rhodotorulaceae, produces brightly colored pigments. Various species in this group, particularly red, pink, or salmon-colored varieties, have been described as agents of spoilage in various organic materials and uncooked foods like canned oysters.[7] They are found as nuisances in places where foodstuffs are prepared, as in butcher shops and oyster-shucking establishments. They are not pathogenic. Most of the torulas have little fermentative ability and consequently are of little commercial value.

Several pathogenic genera of fungi are classified near the torulae, especially certain ringworm and dermatitis fungi, as indicated in the table.

Pathogenic Action of Yeasts. A number of diseases, some of them very serious, are caused by certain yeastlike fungi. Since these also produce definite mycelial growth they are discussed in the chapter on Molds (Chapter 22).

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Chapter 22

THE MOLDS

The term "mold" is a general one and, strictly speaking, has no exact definition. It is a convenient term, however, and for present purposes may be taken to include most of the woolly, cobweb-like, cottony or powdery growths, black, green, yellowish, or white, commonly seen on stale bread, or on old piles of manure, or on books or shoes in the summertime when the humidity is high. We shall also include certain mycelium-producing organisms which have some yeast-like characters. For a good general discussion see Wolf and Wolf.[1] Molds are common on the tops of jams and jellies which have been imperfectly sealed and have stood for a long time. Their spores are as ubiquitous as those of bacteria. They are able to grow in situations where bacteria cannot survive because of high osmotic pressures, acidity, low moisture content. They are characteristically strict aerobes and thus cannot compete with anaerobic microorganisms. In general, their metabolic activities are much like those of bacteria. Foster has written an interesting survey of their methods of dissimilating various compounds. The process is much like that found in bacteria.[1a]

Structure. The woolly growths (called *mycelia*) consist of more or less compact masses of intertwining, branching, hairlike filaments called *hyphae,* which grow up into the air. In many species, when circumstances permit, there are also developments of subsurface hyphae within the material on which the mold may be growing. These serve to anchor the plant but are not otherwise especially differentiated from the aerial mycelium. The aerial mycelium carries on it the fertile hyphae which form the reproductive organs while the remainder of the mycelium absorbs moisture and food material.

As indicated in the table on page 375, molds corresponding to the general description given above may be found in three large divisions of the Eumycetes; namely, Ascomycetes (group II), Phycomycetes, and Fungi imperfecti. Peculiarities of structure and methods of reproduction enable us readily to distinguish filamentous Ascomycetes from Phycomycetes and we shall discuss these details first, comparing the two groups in a general way.

The *filaments* of molds may consist of elongated cells arranged end-to-end in *mature* growths, and separated by walls (*septa*) as in the Ascomycetes or, as in the Phycomycetes, the whole mycelium may consist of one continuous protoplasm-filled tube which is coenocytic (not divided into sections by septa). In mature plants septation or nonseptation is a fairly stable characteristic and serves as an aid in differentiation among molds. As compared with bacteria, the diameter of mold filaments is relatively large; sometimes 20–30 microns.

The nuclei of molds, within the filaments, are extremely minute, but generally quite definite. In the Ascomycetes each cell contains one nucleus except during phases of reproduction. In the Phycomycetes the nuclei are not separated by definite cell walls except as a result of the formation of spores.

The cytoplasm of molds is granular and contains droplets of fat, carbohydrate and nitrogenous material, including volutin. The cell wall of some species probably consists of cellulose; in others the cell wall seems to be made of chitin-like substance.

Reproduction of Molds. Portions of the mycelia of molds, when transferred to new media, will usually continue growth much as many other plants do, the hyphae continuing to elongate, usually by growth at the ends. After a time *fertile* aerial hyphae are produced which begin the process of multiplication by formation of *asexual* spores (*conidia*). The color of mold colonies, when present, is often due to the asexual conidia, which are borne in enormous numbers on the fertile hyphae. Asexual conidia are not in asci, being formed merely by subdivisions of fertile hyphae.

Spores formed by processes analogous to *conjugation* or sexual fertilization also occur in some species. For example, in the Ascomycetes, two morphologically *different* cells unite to form the sexual spores. The spores thus formed are enclosed in sacs or asci and are called *ascospores* (hence, Ascomycetes).

In the Phycomycetes sacs are not formed, but separate and free sexual spores result from the fusion of morphologically *similar* or *dissimilar* cells. Often the cells which fuse to form these zygospores, as in *Rhizopus,* are on two separate plants, which produce the spores wherever hyphae come into contact, suggesting that the plants are of opposite sexes. Neither plant alone produces zygospores. Such plants are said to be *heterothallous,* and their "sexes" are called + or —.[2]

Fungi imperfecti are those (chiefly much like the Ascomycetes) in which no sexual spores have been observed. This, however, does not prove their absence and, as Henrici points out, the imperfection about *Fungi imperfecti* is largely in our knowledge of them. Many produce conidia or asexual spores.

Further details of reproductive methods will be taken up later in connection with descriptions of individual species.

Study of Molds. An excellent method of cultivating yeasts and molds for demonstration of their gross morphological details, pigment, etc., is in the form of giant colonies.

Appropriate culture medium (*e.g.*, Sabouraud's) is poured into flasks or bottles of about 200 ml. capacity, to a depth of about ½ inch, sterilized and allowed to solidify. This gives a fairly broad surface and the volume of agar serves as a moisture reserve. A speck of inoculum is transferred to a point in the center of the agar. It is well to do this with the bottle or flask in an inverted position to avoid contamination by dust. The vessel is then plugged with a rather tight cotton plug and held at 25° to 30° C. for some days. Giant colonies grow out, showing various details of color, form, etc., characteristic of the different organisms.

Material from giant cultures in dishes may be examined with low power lenses *in situ,* or portions may be teased out on a slide in a drop of mounting fluid* and examined under a cover slip. Drying produces shrinkage and distortion, while manipulation, such as teasing out, causes fracturing and loss of conidia.

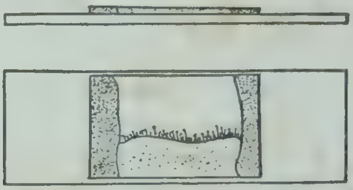

Fig. 164. Method of growing molds between a slide and cover slip. (Reprinted by permission, from Henrici, "Molds, Yeasts, and Actinomycetes," by John Wiley & Sons, Inc., publishers.)

A good medium for the primary isolation of fungi, especially dermatophytes (fungi found in skin diseases), is a modified Sabouraud's agar containing substances inhibitory for many bacteria of the skin, which usually grow faster than fungi. It contains, in water, 1 per cent each of peptone and dextrose; 1.5 per cent oxgall; 2 per cent of agar; crystal violet in a concentration of 1 in 100, 100,000; and 30 units of streptomycin per ml. of medium. The *p*H is not adjusted.[3]

For the microscopic examination of molds the slide culture is an excellent method. A good arrangement is to cement a sterile cover slip to an ordinary (previously sterilized) slide with two other bits of glass arranged so that the cover slip is raised about 1 millimeter above the slide. The desired nutrient agar, melted, cooled to about 45° C. and inoculated with spores, is admitted between the slide and the cover slip and the culture incubated (Fig. 164). This permits examination of the growth with fairly high power lenses and in a living, undistorted condition. The best conditions of incubation for most molds are a moist atmosphere and a temperature around 30° C. There should be free access of oxygen. The best *p*H is around 5.2 but some molds will grow in the presence of fairly strong (2.5N) acid.[3a]

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* A useful mounting fluid for fungi consists of a mixture of equal parts of glycerin, ammonium hydroxide and ethyl alcohol.

A special type of slide-culture vessel has been devised by Brown[4] for the same general purpose. This is shown in Figure 165.

Method of Use. The slide is sterilized by passing it through a flame. If agar is to be used, the slide should be placed on a warm stage or on a hot water bottle. Small droplets of mineral oil are placed at the six points marked "x" in Figure 165, *B*. A large loop of the inoculated medium (for example, Sabouraud agar) is placed on the central stage at the spot marked "O." The preparation is then carefully covered with a flamed 25-mm. cover glass which should cause the inoculated medium to spread out in the form of a broken circle on either side of the notch at the edge of the stage, which notch serves as an air inlet. The slide may now be incubated in a Petri dish containing a piece of moist filter paper to prevent drying.

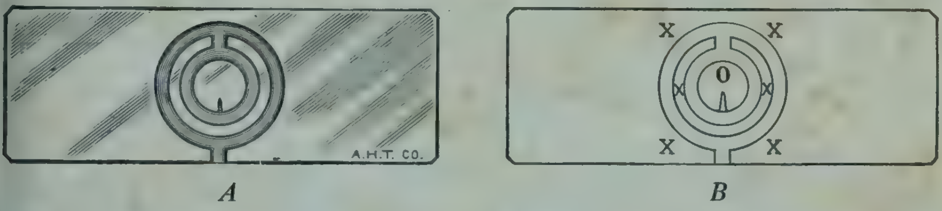

Fig. 165. Special slide for cultivation of fungi. (Brown). The inoculated medium is placed upon the stage and covered with a thin cover glass and sealed with oil. Air can reach the culture only through the moats, which delays evaporation of the culture medium and results in minimum danger of contamination. The design of the slide also permits a thin layer of medium so that the aerial and deep growth can be observed under high magnification.

If it is desired to arrest growth of the culture at any stage and to make a permanent preparation, place the slide culture in a desiccator over formaldehyde solution for several hours, and then seal off the exterior air inlet, *i.e.,* short, straight moat extending from edge of slide o outer concentric moat, with paraffin by means of a hot spatula. The culture can be observed at any time under the dry objectives of the microscope. Structures near the cover glass can be observed under the oil immersion objective.

A simplified slide culture technique requiring no special apparatus as been described by Johnson.[5] It is based on the method described on page 390.

Habitat of Molds. Mold conidia are to be found in all dust, as every ousewife knows who has had preserves spoiled by them.[6] They occur on decomposing organic matter like manure piles, "compost" heaps, dead plants and animals. They can thrive especially under conditions where there is too little water for the active growth of yeasts or bacteria. For example, they grow as "mildew" on books, old shoes, clothing and floors in damp weather.

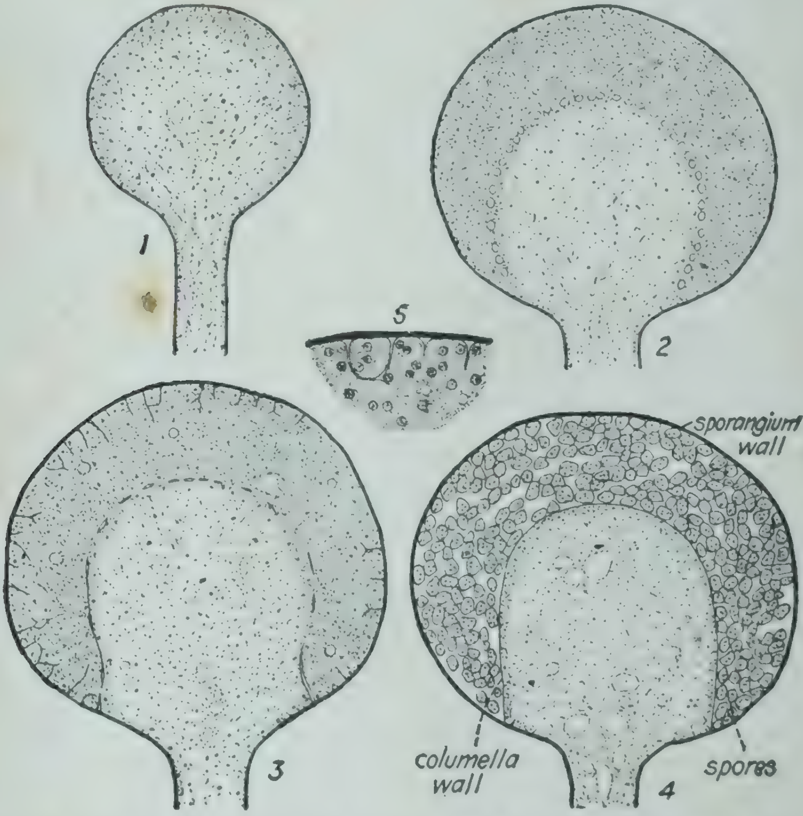

Fig. 166. One of the Mucorales. Stages in formation of a sporangium filled with spores or conidia. *1*, Sporangiophore and columella; *2*, multiplication of spores from cells of the columella, and formation of the retaining membrane; *3* and *4*, completion of sporangium formation. (From Swingle's Plant Life. D. Van Nostrand Co., Inc.)

Physiologically they are, as a group, active enzyme producers, causing very rapid oxidations and decompositions of carbohydrates, fats and proteins, and other biochemical changes in a great variety of substances. They are of great commercial value in the production of certain enzymes and various organic compounds which are used as foods, flavors and drugs. From carbohydrates they produce hundreds of substances not easily made by artificial processes, and of great usefulness. Among these are penicillin, etc., acetone, butanol, sorbitol, takadiastase, and so on.[7] This is an excellent field for research.

I. THE PHYCOMYCETES

The group of Phycomycetes contains several genera, two of which are very commonly observed, and often very troublesome, namely, *Mucor* and *Rhizopus*. These are placed in a group called Zygomycetes because they form zygospores (see Fig. 167).

Genus Mucor. This genus contains several dozen species, many of them very similar to one another. One of the best known of these is *Mucor mucedo,* a coarse, woolly, white mold seen on piles of rotting manure or other decaying organic matter. It is active in the chemical decomposition of fats, proteins and carbohydrates.

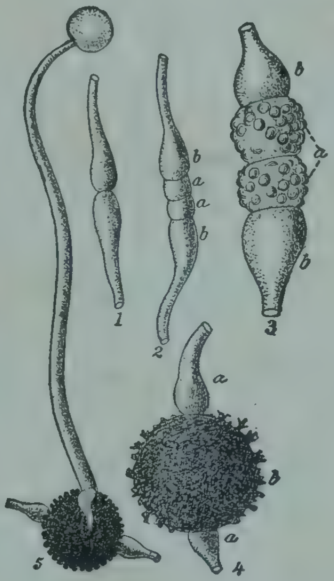

Fig. 167. Successive stages of zygospore formation in *Mucor mucedo.* (From Jordan, after Brefeld.)

Mucor mucedo forms large numbers of submerged hyphae and reproduces asexually by means of *sporangia,** filled, as a rule, with black spores. Each sporangium is borne on a short *sporangiophore*† (compare *conidiophore* in the filamentous ascomycetes) at the tip of which is an enlarged portion called a *columella* which varies in shape according to the species (Fig. 166, *1*). The spores are formed in a mass about the columella within the envelope (Fig. 166, *2, 3, 4*).

During sexual reproduction the tips of two heterothallic hyphae touch each other (Fig. 167, *1*) and grow into contact, the apical portions of each being segregated by septa and swelling to form larger bodies, the *gametes* or sexual cells (Fig. 167, *2a* and *3a*). These fuse, resulting in a cell called the zygospore (Fig. 167, *4b*). This goes into a "resting stage" of greater or less duration and eventually, finding conditions of moisture, temperature and nutrition favorable, bursts or germinates, sending forth a hypha which may be either fertile, giving rise at once to a sporangiophore with a sporangium at the tip, as shown in Figure 167, *5*, or vegetative. *Zygospore formation is limited to the yeasts and Phycomycetes.* Mucors and related genera of molds are common contaminants of bacteriological cultures. A few have been found as the cause of disease in man and animals.

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* A sac or receptable in which *asexual* spores are produced (distinguish from *ascus* and *perithecium*).

† A stalk on which the sporangium is borne.

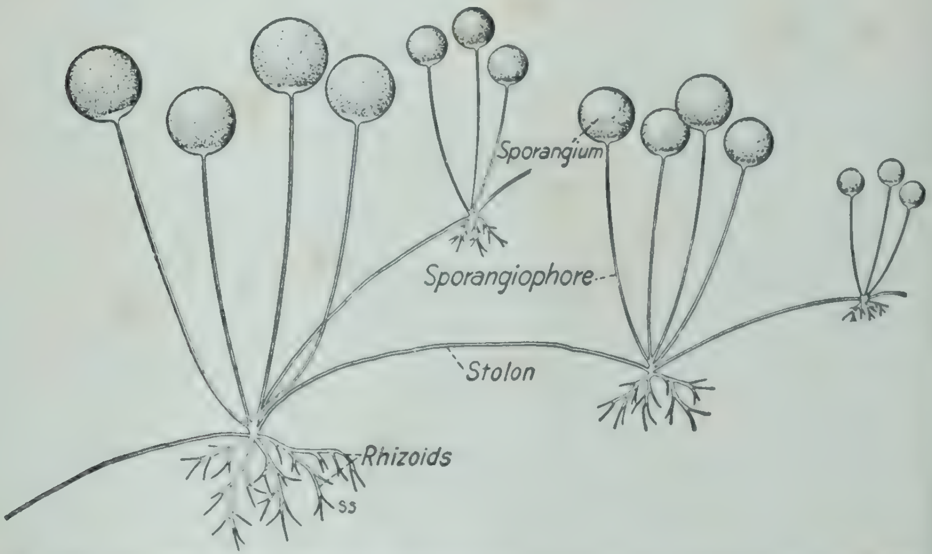

Fig. 168. *Rhizopus nigricans.* Plant spreading by stolons. (From Swingle's Plant Life. D. Van Nostrand Co., Inc.)

Chlamydospores. As in the yeasts, cells of *Mucor, Rhizopus,* and related species often become rounded, acquire a thickened wall and go into a quiescent state somewhat resistant to drying, sunlight and other mildly unfavorable influences. Such cells are often seen distributed along filaments of the molds.

Genus Rhizopus. The molds of this genus are well exemplified by *R. nigricans,* the common, black, bread mold familiar to all who have seen bread after it has stood in a humid place for some days during the summer. It spreads rapidly because it sends out stolons or runners (Fig. 168) like some kinds of grass ("crab grass") and strawberry plants. These runners take hold of the substrate by means of "holdfasts" or rootlike hyphae.

Like *Mucor mucedo* and its allies, *R. nigricans* is an active enzyme-producer and is prominent in bringing about decomposition and spoilage of various fruits, vegetables, and other organic materials.

Molds of the genera *Mucor* and *Rhizopus* are similar in many respects but may be easily differentiated by at least three characters as follows:

	Mucor	*Rhizopus*
Runners	Absent.	Present.
Sporangiophores	May arise at any point in the mycelium.	Arise only at the "holdfasts" where the runners touch the substrate.
Columella	Never hemispherical; continuous with the sporangiophore.	Hemispherical; a single, differentiated cell.

II. THE FILAMENTOUS ASCOMYCETES

The group of Ascomycetes as a whole contains many widely differing plants, varying from the yeasts, through the filamentous woolly molds, to fleshy mushroom-like morels and the cup-fungi which grow on rotting logs. All have in common the formation of asci with ascospores and all are modifications or evolved patterns of the same basic growth design. The yeasts have already been described. As morels and cup-fungi have no relationship to bacteriology, they will not be discussed here. This leaves some of the common filamentous Ascomycetes for our attention.

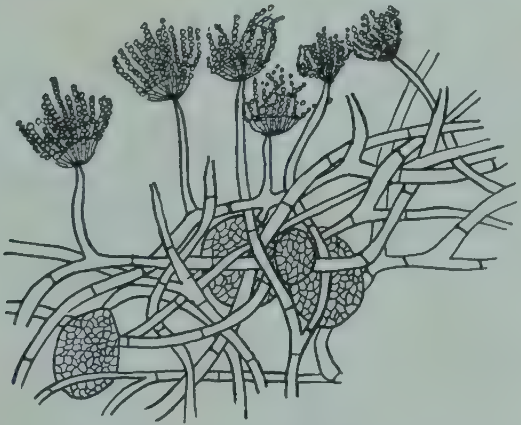

Fig. 169. Species of *Aspergillus* showing structure and arrangement of conidiophores, mycelium and perithecia.

The filamentous Ascomycetes, especially the common genera *Aspergillus* and *Penicillium*, are distinguished from the *Mucor* and *Rhizopus* molds in: (1) having septate mycelia; (2) forming sexual spores in sacs (ascospores) instead of single and free like zygospores; (3) not

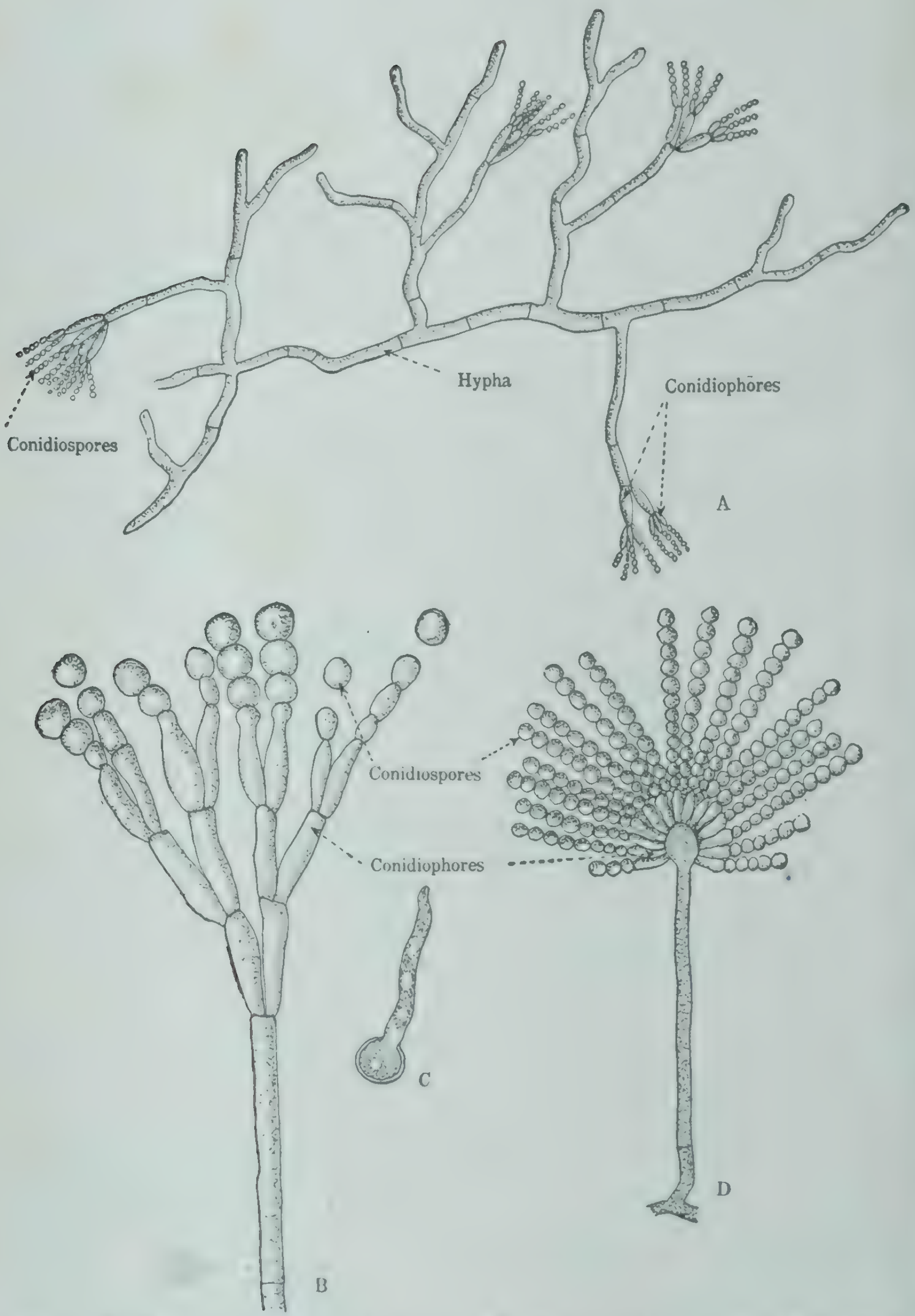
Conidiospores
Hypha
Conidiophores
A
Conidiospores
Conidiophores
B
C
D

Fig. 170. Blue and green molds. *A* and *B*. Common blue mold (*Penicillium*); *C*, germinating conidiospore; *D*, green mold (*Aspergillus*). (Reprinted by permission, from Holman and Robbins, "Textbook of General Botany," John Wiley & Sons, Inc., publishers.)

producing conidia enclosed in sporangia, but free in groups of various sorts such as chains, masses of characteristic shape, etc. (Figs. 169, 170).

Two of the best known genera are *Aspergillus* and *Penicillium.* It must be pointed out that, although we discuss sexual processes of *Aspergillus* and *Penicillium* at this point, the sexual structures of most species are unknown and *Aspergillus* and *Penicillium* are therefore important genera in the group of Fungi imperfecti (see page 375).

Genus Aspergillus. During sexual reproduction a fusion of certain homothallic cells* results in the formation of hollow, flasklike or gourdlike bodies called *perithecia* which contain asci with spores (Fig. 169). It is for this reason that the aspergilli are included in the same order (Ascomycetes) as the yeasts.

Perithecium Formation. In the formation of a perithecium two hyphal cells twist about each other. The separating walls dissolve and the resulting fusion-cell gives rise to a nodule of branched hyphae among which a mass of asci or spore sacs is formed. The whole mass is often enclosed in a sort of hull or protective cellular covering formed by the surrounding mycelium. Each ascus, as a rule, contains eight ascospores.

The asexual fruiting bodies, or conidiophores, of aspergilli consist of tall hyphae which have enlarged globular tips. From the surface of these project numerous small stems called *sterigmata,* and on the tips of these the conidia are borne in long chains (Fig. 170, *D*). There is no sac or envelope as in the zygomycetes. Classifications of aspergilli are based to some extent on the structure, but more particularly on the color, of the conidial heads.

Common Species. One of the commonest species of *Aspergillus* is *A. glaucus.* It forms green or grey-green conidia and yellow perithecia and is frequently seen on bread, preserves and clothing during the summer or in the tropics. Another common environmental species, sometimes found as a laboratory nuisance, *A. nidulans,* is recognized because of its bright green color. The perithecia are reddish in color. *A. niger,* also an extremely common species, is recognizable by its very large round masses of black conidia. It is sometimes confused with *Rhizopus nigricans* which produces black sporangia.

There are some pathogenic species. For example, a lung infection of birds due to *Aspergillus fumigatus* is not uncommon. However, the molds of the genus *Aspergillus* are chiefly of importance as scavengers and are found, usually along with other molds and bacteria, busily engaged in the decomposition of a great variety of waste matter. On the other hand, due to this ability to decompose organic matter

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* Cells on the same plant.

Fig. 171. *A, Penicillium sp.* The rapidly growing colony (50 mm. in diameter, eight days) is at first white, then becomes bluish-green and very powdery due to abundant spore production from the aerial mycelium. (Conant, Smith et al., Manual of Clinical Mycology.)

B, Penicillium sp. Spore-bearing hyphae characteristically form a "Penicillus" or brush. The conidia occur in unbranched chains (e) cut off from the tip of flask-shaped sterigmata (d) which are verticillately arranged (in whorls) from the ends of metulae (c) arising from branches (b) of the conidiophore (a).

Although species of *Penicillium* differ in gross appearance (size of colony, color, texture, etc.), the genus may be identified by the characteristic structure of the conidiophore which arises from vegetative hyphae either in, on or above the agar at the end of which branching takes place to form the typical "Penicillus" or brush.

C, Paecilomyces sp. The rapidly growing colony (70 mm. in diameter, five days) thinly covers the agar surface and becomes yellowish-brown and powdery due to an abundant production of conidia.

D, Paecilomyes sp. The conidial-bearing hyphae superficially resemble the "Penicillus" of *Penicillium*. Elliptical conidia (d) occur in unbranched chains cut off from the tip of flask-shaped sterigmata (c) which not only appear verticillately arranged (in whorls) on the ends of hyphae, but also a single sterigma may appear along a hypha (a). The appearance of the single sterigma (a) from the hyphae and its characteristic taper into a long, conidial-bearing tube (b) which bends away from the main axis of the sterigma and the accessory cells or "macrospores" (e) found in or close to the surface of the agar distinguish this fungus from those of the genus *Penicillium*.

E, Scopulariopsis sp. The slow growing colony (60 mm. in diameter, eighteen days) is at first membranous, wrinkled and glabrous until aerial hyphae and conidia develop to give the culture a powdery, light brown appearance.

F, Scopulariopsis sp. The conidia-bearing hyphae cluster to resemble superficially the "Penicillus" of *Penicillium*. Sterigmata, bearing unbranched chains of rough-walled conidia (c), may be clustered from branches of a short conidiophore (a) or appear singly along the aerial hyphae (b). The conidia are characteristically lemon-shaped with a pointed apex and truncate base (d).

Although species of *Scopulariopsis* have been reported from nail infections and from deep-seated granulomatous lesions, they are common contaminants and must be investigated most carefully before being reported as an etiologic agent of disease.

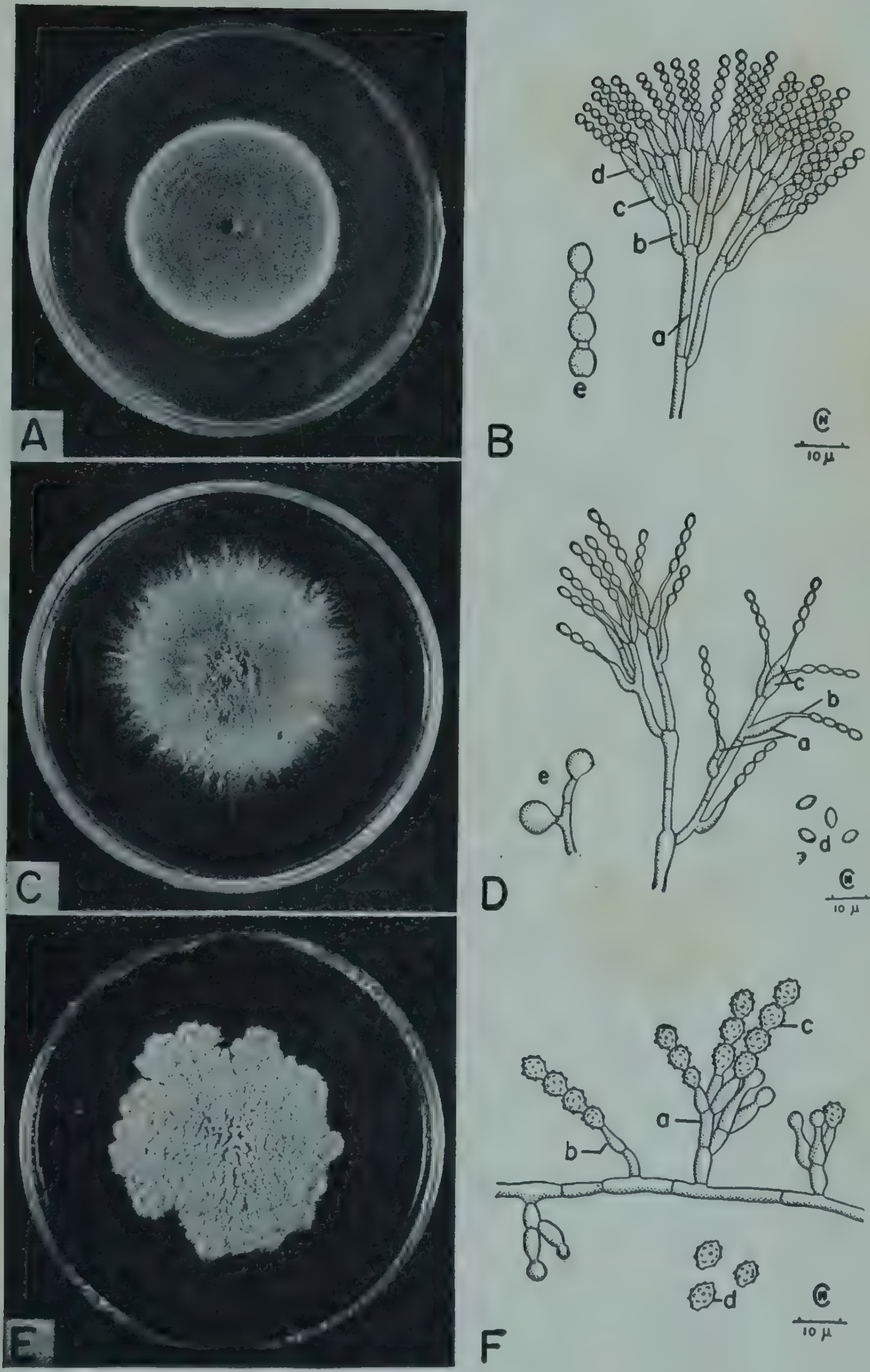
d
c
b
a
e
A
B
10 μ
c
b
a
e
d
C
D
10 μ
c
a
b
d
E
F
10 μ

Fig. 171.

these organisms are of great importance as nuisances and cause much spoilage of food, wood, paper, and other commodities composed of organic matter. The well-known "moldy" odor characteristic of some damp cellars is due in part to the growth and metabolic products of these organisms (and also in part to actinomyces).

Genus Penicillium. The penicillia are very common in Nature and contribute to the spoilage of various objects and materials composed of organic matter, especially ripe fruits. Like the aspergilli, the mycelia are septate in structure.

The conidiophores are composed of hyphae which branch extensively at the tip into clusters called *verticils*, the whole roughly suggestive of the bony structure of the hand. The spores extend in parallel chains from the ends of the "fingers" (sterigmata). This arrangement gives the whole conidiophore a form suggestive of a tiny paint brush, from which the generic name is derived (Fig. 170, *A* and *B*). As in other groups of molds, the color and form of the fruiting body furnish characters of differential value in classification. Sacs or perithecia are formed by some penicillia in much the same manner as by the aspergilli, but most species of *Penicillium* are classed as Fungi imperfecti.

Some species of *Penicillium* are differentiated chiefly by their habitat. Thus, the green molds found in Roquefort cheese (*P. roqueforti*), Camembert cheese (*P. camemberti*) and other cheeses of the same nature are distinguished chiefly because of their occurrence there. The molds grow in or on the cheese, producing various enzymatic changes in the fat, carbohydrate and protein of the cheese which result in characteristic aromas, flavors and textures. *P. roqueforti* grows well under conditions of temperature and humidity found in the limestone caverns in the province of Roquefort, France. It is microaerophilic, growing in the interior of the cheese masses provided holes are punched in the cheese to admit small amounts of air. (These holes must not be confused with the holes in Swiss cheese produced by gas formed by the *Propionibacter*.)

Penicillium camemberti, which closely resembles *P. roqueforti*, is strictly aerobic and grows only on the exterior of Camembert cheese, so that the ripening of this delicacy proceeds from without inward (see page 675).

There are many other species of penicillia and they are frequently seen on old bread, cheese, lemons, and other fruits. They may usually be recognized as members of the *genus* by their sky-blue or green color. The individual *species* may be differentiated by the various arrangements of the conidiophores.

Giant colonies of some common molds resembling *Penicillium* are seen in Figure 171.

Penicillium notatum and *P. chrysogenum,* very similar species, have come into great prominence as sources of one of the best drugs for use in infections of the blood and tissues. The active principle, called *penicillin,* is given off as a waste product into ordinary medium during growth. It is more fully discussed in the section on disinfection (see page 240).

Some aspergilli are sources of antibiotic substances similar to penicillin, *e.g., A. clavatus* produces *clavacin.*

Fungi Imperfecti. The Fungi imperfecti constitute a group of which the reproductive cycle is not known. The membership of the group is, therefore, rather heterogeneous since imperfect forms of Ascomycetes, Phycomycetes, Basidiomycetes, etc., are numerous. The largest number of species of Fungi imperfecti, however, are really Ascomycetes of which our knowledge is incomplete.

Classification of Fungi imperfecti must be based on asexual structures and for this reason genera of Fungi imperfecti are not necessarily composed of related species. Inasmuch as there are about 1,330 genera and some 20,000 species, a complete discussion of the Fungi imperfecti here is out of the question. We may, however, give attention to a few common species of filamentous fungi of this group which any bacteriologist may from time to time encounter in his laboratory work.

I. *Sporotrichum.* Conidia oval or pear-shaped, occurring singly or in clusters at all parts of the mycelium; attached by pedicles or stems; never in chains; never on conidiophores.

II. *Trichoderma.* Conidia occur in compact masses on tips of well-developed conidiophores which branch like trees or shrubs; bright green in color; common in soil and as contaminant in laboratory.

III. *Oidium,* Monilia, Candida, etc.* A large group in which confusion exists as to nomenclature and classification, especially between medical mycologists who use the terms *Oidium* and *Monilia* rather unsystematically, and general or industrial mycologists who have developed other terminology and taxonomic systems. In the main, these organisms are neither clearly filamentous molds nor are they exclusively yeastlike, but partake of the characters of both. The formation of frank, mold-like filaments is not a common occurrence; the yeast-like characters are more often seen.

Following the lead of Skinner, Emmons and Tsuchyia[9] the group may be divided into three genera based upon mode of reproduction: (1) *Geotrichum,* reproducing by fragmentation or disarticulation of the mycelium into separate cells called *arthrospores;* (2) *Candida,* reproducing by the development of yeastlike cells, or oidia, budding from the ends or sides of the mycelium, called *blastospores;* (3) *Trichosporon,* reproducing by both methods. The organisms of the genus *Candida* have often been called *Monilia* by medical workers. Some of these organisms also produce chlamydospores. There seems little doubt that many species of yeasts and molds have at times been wrongly placed in the illy defined group of *Monilia* or *Oidium,* especially by medical writers. An excellent review of the situation has been written by Skinner,[9a] who points out the advisability of using *Candida,* and not *Monilia.*

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* Oidium is from a Greek word meaning "little eggs," and is derived from the shape of the organisms.

IV. *Aspergillus.** Round or oval conidia produced in unbranched chains from sterigmata which radiate from a central, unbranched, club-shaped conidiophore, new conidia formed at *base* of chain.

V. *Penicillium.** Round or oval conidia produced in unbranched chains from clustered, digital branches of the conidiophore; new conidia formed at *base* of chain.

VI. *Hormodendrum* or *Cladosporium.* Conidiophore produces one conidium, which reproduces *apically,* branching as the chains of conidia form.

VII. *Alternaria.* Conidia contain 8 to 12 or more large cells in a conical group, the divisions between the cells being in various planes; the groups occurring in chains, or spaced along the mycelium.

Genus Sporotrichum. There are several saprophytic species of *Sporotrichum,* among which *S. schenckii* is of particular interest because, in addition to living a saprophytic existence, attached to grasses and other plants, it also causes a disease of man known as sporotrichosis.

In the latter circumstance it probably gains entrance to the skin or mucous membranes and lymphatic spaces by way of wounds made by straw, thorns and similar objects. It grows along the lymph spaces, producing rows of red nodules which sometimes ulcerate. There is little pain or soreness. Sporotrichosis lesions superficially resemble those of tertiary syphilis and special care should be taken in areas where sporotrichosis occurs, to make certain of the diagnosis. In the pus, when stained by Gram's method, the organisms are seen in the pus cells as elliptical, gram-positive bodies about the size of some bacteria of the genus *Bacillus* (2 μ x 8 or 10 μ). The disease may also occur in animals.[10]

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* Most of the *Aspergillus* and *Penicillium* species are Fungi imperfecti. As these genera have already been described they are not discussed here.

Sporotrichum may be isolated from such material on one of the media adapted for the growth of molds, preferably Sabouraud's agar. The colonies resemble those of bacteria, but contain mycelial filaments closely packed. They turn brown with age. Aerial mycelia are seldom formed. The forms of conidia and hyphae are seen in Figure 172.

Genus Trichoderma. The best-known species of this genus is *T. koningi* or *T. viride,* a common soil and environmental species which often enters laboratory cultures uninvited. It is of a bright green color with well-developed aerial mycelium (Fig. 173). It forms large amounts of ammonia, and an ammoniacal odor gives a clue to its identity.

Genera Candida, Trichosporon, Geotrichum. As indicated above, some of the organisms of these genera are moldlike in that at times they form mycelial growths while at other times they form yeastlike cells, the two sorts of cells sometimes occurring simultaneously in the same culture or pathological lesion. Many species are known, some of them being common contaminants in the laboratory, but only three will be mentioned here.

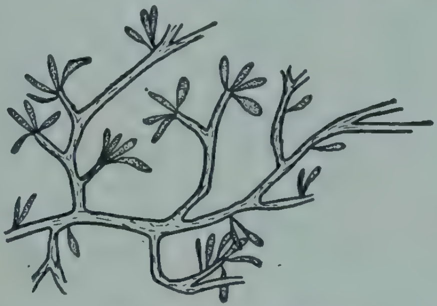

Fig. 172. *Sporotrichum schenckii.*

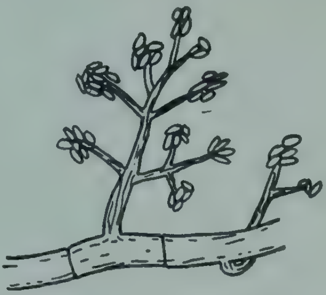

Fig. 173. *Trichoderma koningi.* (Redrawn from Henrici.)

One representative species is the harmless *Geotrichum candidum,* often called *Oidium lactis.* This readily metabolizes lactic acid and is of common occurrence in soured dairy products, like sour milk, cheese or butter, and in sauerkraut and silage where lactic acid is formed by other organisms in these products. Like most other molds, it is metabolically versatile, attacking proteins and many other compounds, producing ammonia and causing spoilage in many fermented products. It grows as a white, felt-like, adherent membrane on the surface of solid media like Camembert and other cheeses, clotted milk or agar. Aerial mycelium forms as the plant grows older, and is distinguished by *dichotomous* branching. Conidia are borne in long chains. Lactic acid is necessary for any extensive growth.

Candida albicans (also called *Monilia albicans* and *Oidium albicans*) is a well-known species commonly associated with, and probably the cause of, thrush, a disease of the mucous membranes of the mouth and gastro-intestinal tract and sometimes of the vagina. There are local inflammation and white, membranous patches probably often confused with diphtheritic membranes. Microscopic examination of scrapings from the affected parts reveals the filaments and yeastlike cells. A somewhat similar disease of the gastro-intestinal tract, sprue, is thought by some to be due to *Candida albicans* or a species very much like it, *C. psilosis*. However, these organisms occur very widely in normal individuals, and their relation to sprue is questioned by many.

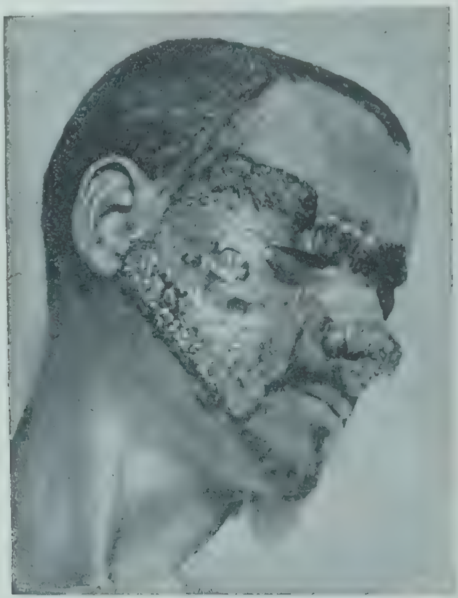

Fig. 174. Blastomycosis due to *Oidium dermatitidis*. (Courtesy of Dr. Isaac R. Pels, from Andrews, "Diseases of the Skin.")

Blastomyces dermatitidis, also widely known as *Cryptococcus gilchristi* and *Oidium dermatitidis,* is a dangerous pathogen, causing the disease known as blastomycosis (Fig. 174). Because of the appearance of large, thick-walled, yeast-like cells, readily seen in pus treated with 20 per cent potassium hydroxide and examined wet under cover slips, the organism was originally regarded as a true yeast. Many clinicians speak of any infection involving a yeast-like fungus as a blastomycosis. The fact that the cells seem to reproduce by budding supported this idea. Later observations of the development, on artificial media, of filamentous growth showed that it belongs nearer to the *Candida* group (Fig. 175).

Factors important in determining whether the mycelial or oidial form shall predominate are temperature (31° C. favoring the mycelial form and 35° C. the oidial form); and *p*H (the oidial form tending to predominate at around 7.5, the mycelial form around 6.0).[11]

Coccidioides immitis, a superficially similar species, is the cause of a disease called coccidioidomycosis occurring chiefly in the San Joaquin Valley of California and elsewhere in arid regions. In nature the organisms live in the soil and their resistant spores are blown about with dust and inhaled. It has been found by Smith,[12] Aronson,[13] and others that a large number of these infections pass unnoticed or result in a mild febrile disease in association with bronchitis, "rheumatism," or pneumonia. The disease is often confused with tuberculosis in x-ray and clinical examinations.[13a]

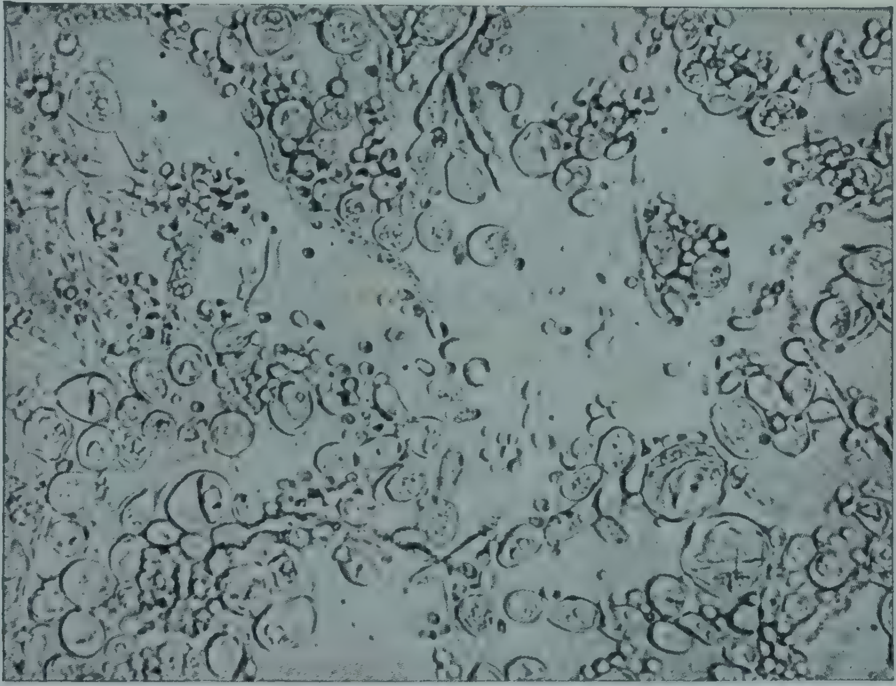

Fig. 175. Unstained, moist preparation of yeast (probably *O. dermatitidis*) cultivated from a case of blastomycosis.

The Coccidioidin Test. A useful means of diagnosis consists in the coccidioidin test, which is based upon the same principle as the tuberculin test (see page 751), *i.e.,* it is an allergic reaction to substances formed by the organism when it is cultivated in broth. The test is performed by the intradermal injection of a minute quantity of the coccidioidin.[13b]

The value of the test was shown in connection with training of troops in the San Joaquin Valley during World War II. By applying the test it was possible to define the areas and seasons most dangerous to the troops, because infection rates were maximal during dusty summer seasons in those areas. The value of growing grass over dusty areas, of paving roads, and of oiling was also measurable in terms of decreased rates at which soldiers become coccidioidin-positive.[14, 14a]

Other diagnostic methods are the complement fixation and precipitin tests.[15]

The causative organism resembles *Blastomyces dermatitidis* in some morphological details and in metabolism. However, it is readily differentiated by cultural and microscopic examination, as shown by Stewart and Meyer.[16]

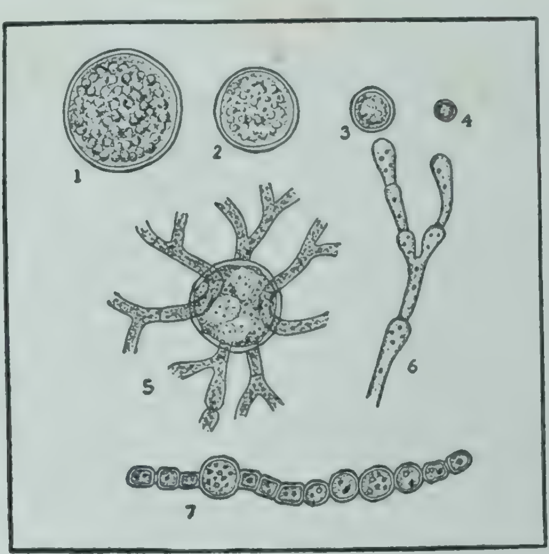

Fig. 176. *Coccidioides immitis* (× 250). *1* to *4*, Cells containing ascospores in tissues; *5*, development of mycelium from large round cell; *6*, mycelium in culture; *7*, old mycelium with chlamydospore. (After MacNeal, Taylor and Moore, from Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company, publishers.)

When *C. immitis* is cultivated in the absence of free oxygen, or when invading the tissues of the body, it forms cells resembling those of *B. dermatitidis,* but they never form buds. On the contrary, the cell contents divide into many smaller cells somewhat suggestive of very exaggerated ascus formation, and the mother-cell wall ruptures, liberating large numbers of the small cells which are then scattered by the blood throughout the body and which repeat the cycle. When cultivated aerobically, mycelial filaments are formed. No conidia are formed (Fig. 176). A good method of cultivation of the organisms occurring in sputum is by the use of an acidified tryptose agar.[17]

When growing in the mycelial form, some of the cells acquire thick protective walls and apparently go into a drought- and sunlight-resistant resting form, like a chlamydospore. It is in this form that the organism is blown about in dry dust, apparently growing vegetatively as a saprophyte in the soil on decomposing organic matter. There is evidence that infection occurs not only through the agency of the chlamydospores but also by the vegetative spherules which occur in the sputum of active cases.[18] The handling of sputum may therefore be dangerous. Cultures are dangerous because of the spores.[12]

Histoplasma Capsulatum. This organism resembles *Oidium dermatitidis* and *Coccidioides immitis* in several respects. It causes infections in man which apparently may pass unnoticed, especially in the young, yet may cause fatal generalized infections in older persons. These infections are characterized by emaciation, splenomegaly, leukopenia, anemia and fever. The infection has been found naturally occurring in dogs. The organism is also pathogenic for rats, mice and monkeys. Encapsulated round or oval cells about 3 microns in diameter may be found in endothelial cells of lungs, liver, spleen, intestine, lymph nodes, skin and buccal mucosa.[18a]

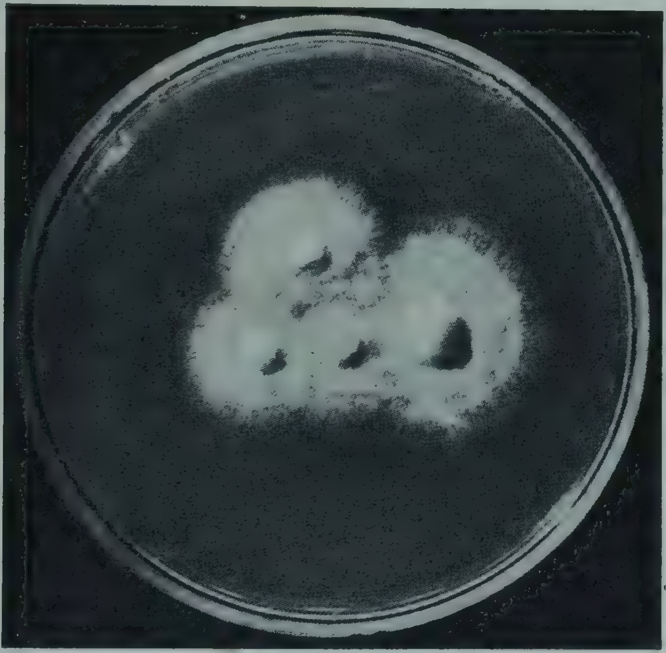

Fig. 177. White cottony growth of *Histoplasma capsulatum* on Sabouraud's glucose agar at room temperature. (Conant, N. F. in J. Bact., vol. 41.)

These organisms may be cultivated on 10 per cent blood infusion agar at 37° C. in tubes sealed to prevent access of air, and loss of moisture. Under such conditions the cells are yeast-like, resembling *Oidium dermatitidis* but differ from it and *Coccidioides immitis* in producing a single bud at one end of the oval cell. In the infected tissues only the yeast-like form is seen.

Like *O. dermatitidis* and *C. immitis,* when cultivated *aerobically* at

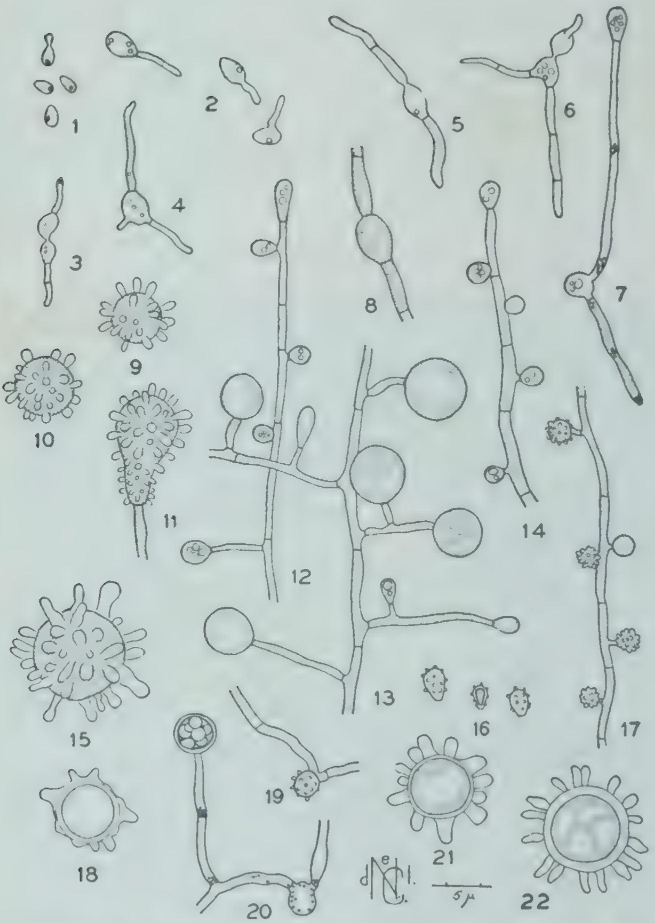

Fig. 178. Life cycle of *Histoplasma capsulatum*. 1. Yeast cells from blood agar incubated at 37° C. 2. Yeast cells germinating with a single tube in 48 hours in Sabouraud's glucose agar cell culture at room temperature. Budding yeast cell germinating from each cell on Sabouraud's glucose agar. 4. Single yeast cell with three germ tubes on Sabouraud's agar. 5. Single yeast cell with two germ tubes on Sabouraud's agar. 6. Budding yeast cell which has germinated with three tubes on Sabouraud's agar. 7, 20. Smooth-filled chlamydospores which have germinated in Sabouraud's broth and produced chlamydospores in eight days. 8. Intercalary chlamydospores on Sabouraud's glucose agar. 9, 10, 15. Round tuberculate chlamydospores from Sabouraud's glucose agar. 11. Pyriform tuberculate chlamydospore from Sabouraud's agar. 12, 14. Small, round, smooth-walled sessile and terminal chlamydospores with oil droplets. 13. Large round smooth-walled chlamydospores on Sabouraud's glucose slide culture in fourteen days. 16. Small pyriform tuberculate chlamydospores from Sabouraud's glucose agar. 17. Small round tuberculate chlamydospores on aerial hyphae. 18, 21, 22. Optical view of thick-walled chlamydospores. 19. Small tuberculate chlamydospore germinating in cell culture. (Conant, N. F. in J. Bact., vol. 41.)

room temperatures on such media as Sabouraud's or glucose agar, a cottony, white, filamentous growth appears. Transferred to 10 per cent blood agar slants in sealed tubes at 37° C. the yeast-like forms appear. The filamentous form is septate and in older cultures there appear large, round, pear-shaped, thick-walled chlamydospores covered with rounded projections. These are especially characteristic of *H. capsulatum*. The colonies of the yeast-like form are small, white, and resemble those of *Staphylococcus albus*[19] (Figs. 177, 178).

Like many infections, such as coccidioidomycosis, etc., occasionally causing death in adults, for a long time only the fatal cases of histoplasmosis were noticed and the disease was thought to have a very high fatality rate. It now appears to be widespread in some sections of the country, notably certain counties of Tennessee, where it causes pulmonary disease which usually heals, leaving scars which are frequently mistaken for lesions of pulmonary tuberculosis in x-ray examinations. In many such persons other tests (tuberculin test; bacteriology) for tuberculosis are entirely negative.

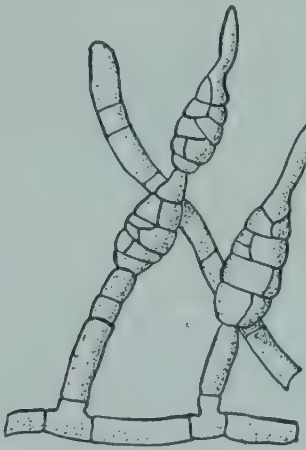

Fig. 179. *Alternaria,* showing muriform multicellular conidia.

As in coccidioidomycosis, extracts of antigenic substances (called *histoplasmin*) derived from cultures of the organism, when injected in minute amounts into the skin, reveal a state of hypersensitiveness to the infecting organism and call forth a reaction (the *histoplasmin reaction*) much like the tuberculin and coccidioidin reactions.[20, 21]

For purposes of preparing histoplasmin a simple fluid medium is very useful. This must contain about 0.175 per cent of agar or other agent to produce slight viscosity. In such medium at 37° C, *p*H about 7.3, abundant filament-free yeast-like growth and histoplasmin production occurs in 2 to 3 weeks.[22]

Genus Alternaria. Several kinds of dark green or brownish-green molds are often met with as contaminants in laboratory media and on Petri dish cultures. Among the commonest forms are members of the genus *Alternaria*. They are not so large and extensive as *Aspergillus* or *Penicillium*. The colonies of *Alternaria* are woolly but are more compact, and the underside and mycelium are very dark colored. The conidia are relatively large, and occur in roughly conical or ellipsoidal masses in which they are packed together so that they are irregularly shaped. These groups of conidia are often arranged in long rows, or occur along the mycelial filaments (Fig. 179).

Genus Hormodendrum or **Cladosporium.** These molds, like *Alternaria,* form relatively small, dark green or brownish-green colonies with a fine, close, velvety surface. They produce conidia by growth at the *tip* of the chains (apically) rather than at the base of the chains as in *Aspergillus* and *Penicillium*. Thus, in contrast to the last two, the youngest conidia in *Hormodendrum* are at the tips. The conidia are rather oval or elongated, and the chains of conidia are often branched (Fig. 180).

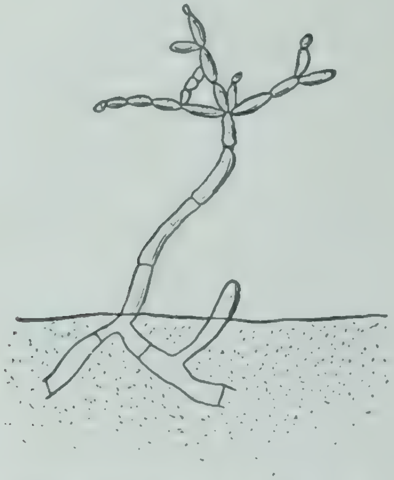

Fig. 180. *Hormodendrum:* dark, smoky or black colonies.

THE DERMATOPHYTES

Occasionally certain species of *Aspergillus* and *Penicillium* invade tissues, especially of the lungs of the human and animal body, producing what are called mycotic infections or mycosis. Such infections are serious and often fatal, but are fortunately rather rare. Mycotic infections of the skin are far more common; in fact skin mycoses are among the commonest of infectious diseases. The molds involved are a rather well-demarked group called *dermatophytes*. In general they belong in the Fungi imperfecti but there are some exceptions.

Ineffective attempts to classify the dermatophytes, naming of new species on the basis of inadequate studies, the great variability of the organisms, their similarity, their occurrence in a variety of different clinical conditions and places on the body and other factors have left the taxonomy and nomenclature of this group in a confused state.

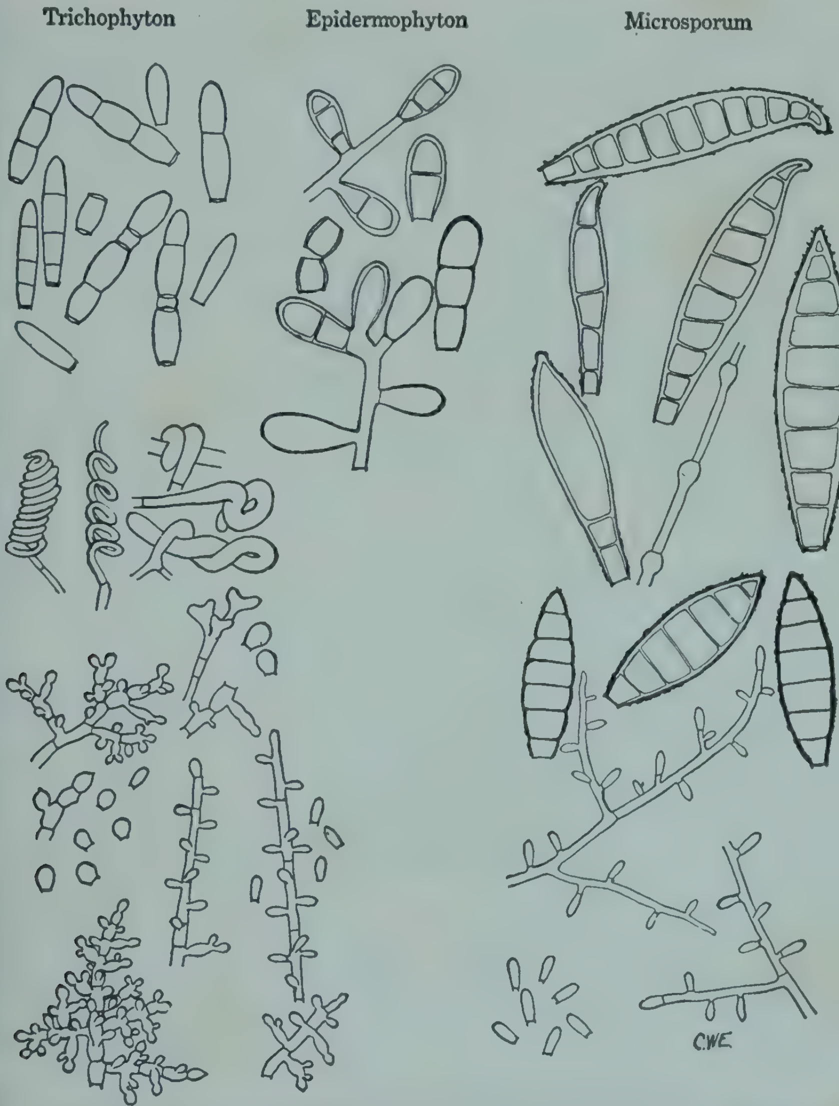

Fig. 181. Spore types in the three genera of dermatophytes. (Skinner, C. E., Emmons, C. W., Tsuchiya, H. M., Henrici's Molds, Yeasts, and Actinomycetes, 2nd ed. John Wiley & Sons, Inc.)

For convenience in this brief discussion we may list some pathogenic fungi as follows:

I. Yeastlike forms.
- Torula (meningitis; European blastomycosis)
- Candida (Thrush; moniliasis; paronychia; vaginitis) (Monilia)
- Blastomyces

II. Filamentous forms.
- A. Ringworm (tinea) fungi.
  - 1. *Microsporum*
    - *M. canis* (animal type; tinea capitis in children)
    - *M. audouini* (human type; tinea capitis in children)
    - *M. gypseum* (animal type; tinea)

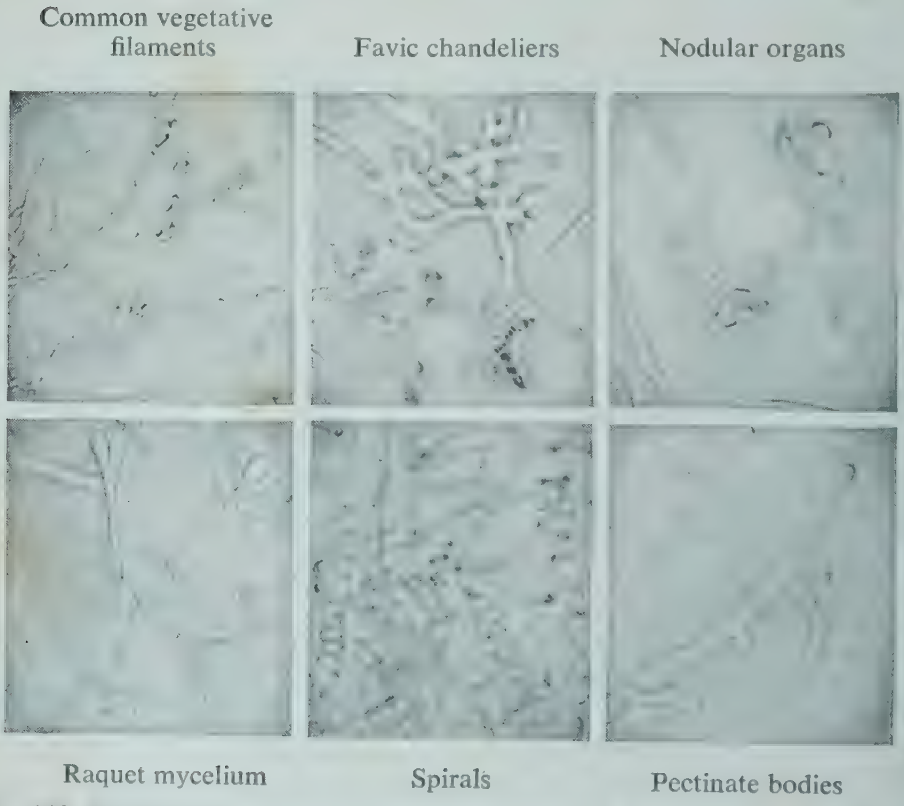
Common vegetative filaments
Favic chandeliers
Nodular organs
Raquet mycelium
Spirals
Pectinate bodies

Fig. 182. Morphologic variants of vegetative forms of dermatophytes. (Seminar, vol. 7, Sharp & Dohme.)

  - 2. *Trichophyton*
    - *T. schoenleini* (Favus)
    - *T. gypseum* (Kerion; tinea sycosis)
    - *T. rubrum; T. rosaceum; T. violaceum* (various forms of tinea, kerion, etc.)
    - *T. tonsurans* ("barber's itch" or tinea capitis)
  - 3. *Epidermophyton*
    - *E. floccosum* (mycosis of hands, feet and groins. Similar conditions caused by various *Trichophyton species*)
- B. Other pathogenic filamentous fungi.
  - 1. *Coccidioides immitis* } Previously
  - 2. *Sporotrichum* } discussed

For a complete discussion of fungus infections the student should consult a textbook on Medical Mycology.[23] Here we may mention two of the commonest mycoses, *tinea* or ringworm, and various mycotic infections of the hands and feet, the last including the various conditions collectively known as "athlete's foot."

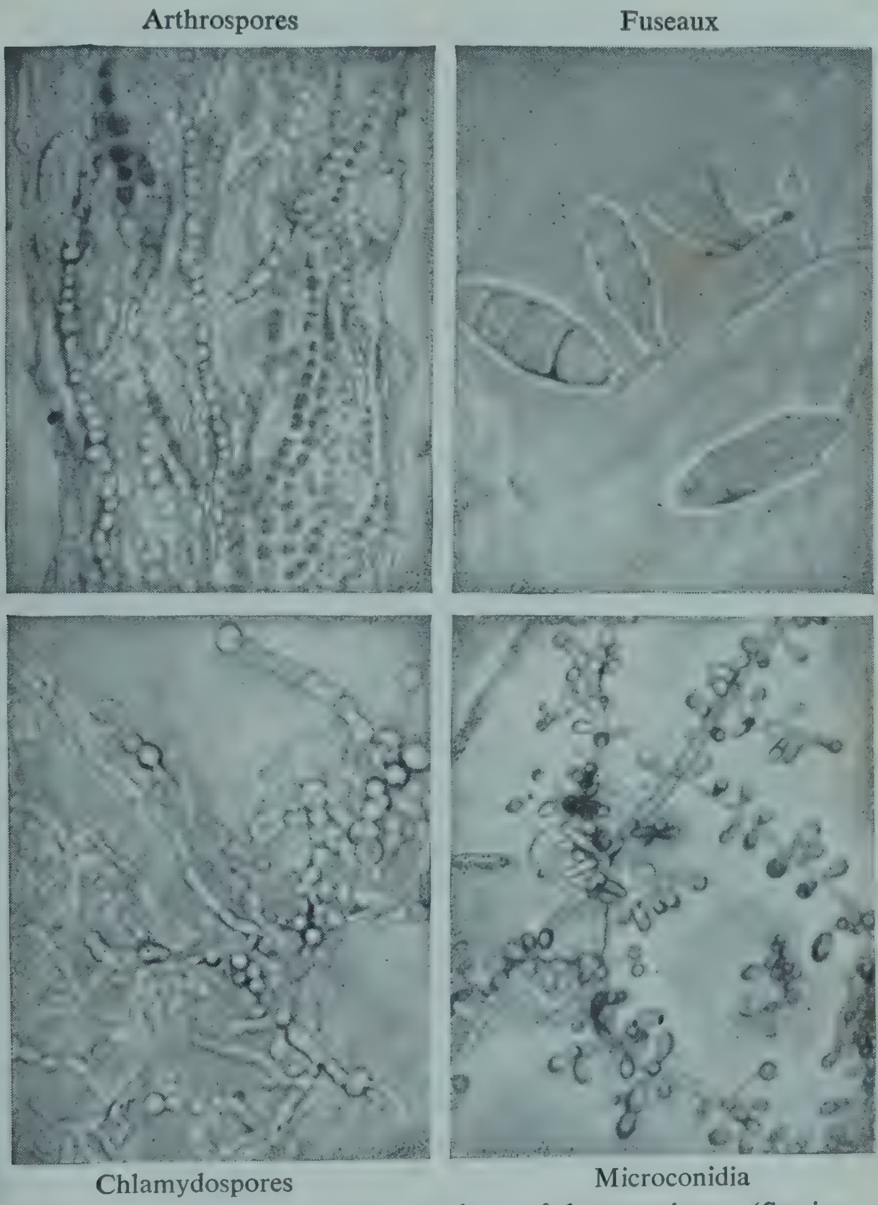

Fig. 183. Morphologic variants of spore form of dermatophytes. (Seminar, vol. 7, Sharpe & Dohme.)

In examining suspected material from tinea capitis (scalp ringworm) stumps of hairs and contents of abscesses are observed. In tinea of the smooth skin, or in mycosis of the hands and feet, scrapings of the affected skin are taken around the margins or from the inside of the covering of vesicles. The material is placed on a slide in a drop of 25 per cent potassium hydroxide, covered with a cover slip and warmed gently for a minute. It may be examined after about twenty minutes. Permanent preparations may be made by washing and staining the material. Cultures may be made on Sabouraud's medium or one of many modifications of it.

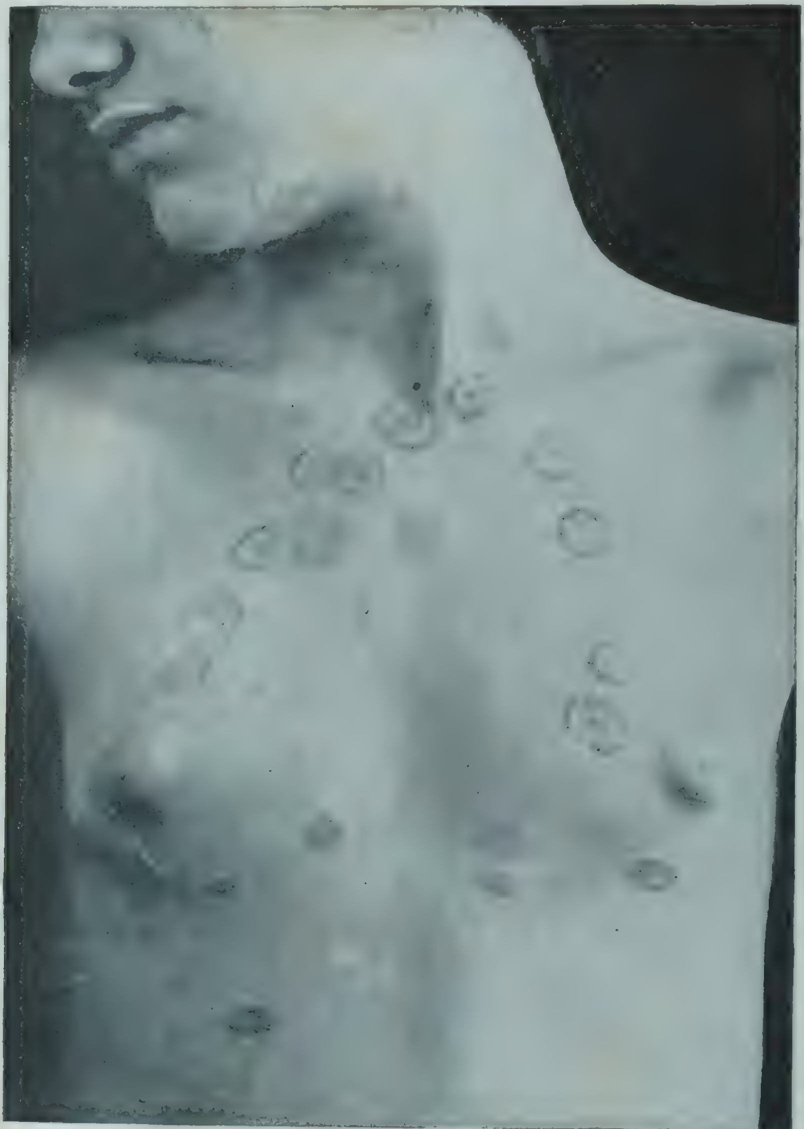

Fig. 184. Tinea circinata. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)

Because of the variable appearance of the dermatophytes in artificial cultures it is difficult to identify them with certainty by such criteria. One of the most constant features is the appearance of large structures called macroconidia. These are found in well-grown cultures one to two weeks old. Other reproductive cells and mycelial peculiarities help to differentiate three of the most common genera—*Trichophyton, Epidermophyton* and *Microsporum.* Some of these are shown in Figures 181, 182 and 183.

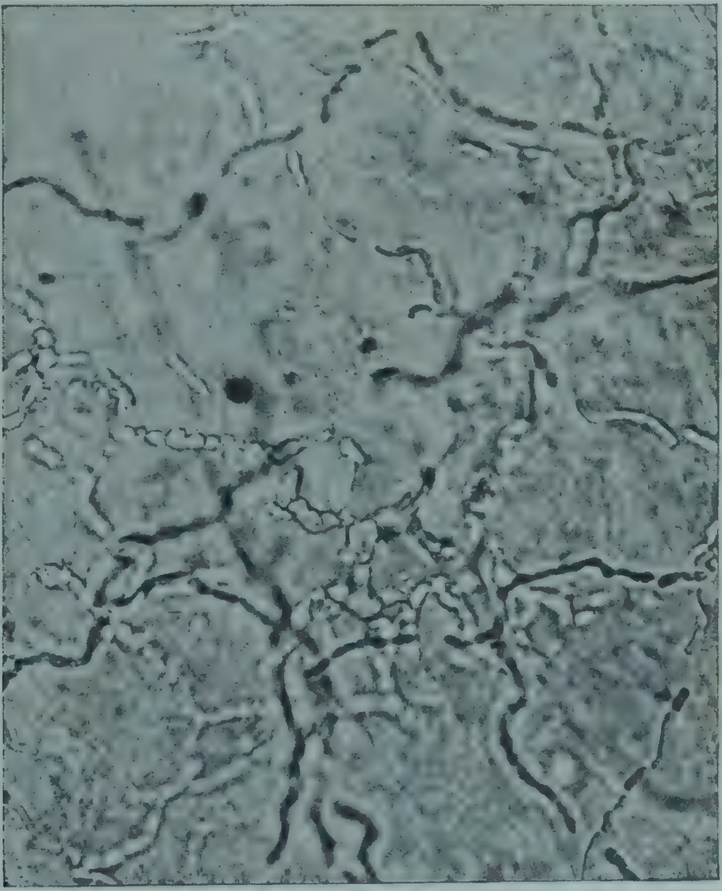

Fig. 185. Mycelia and mosaic structures in scales. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)

Tests for the presence and identity of various dermatophytes include fermentation tests and examination by ultraviolet light (Wood's light). In a dark room ultraviolet rays directed on suspicious patches on scalp or elsewhere will fluoresce due to the fluorescent properties of the fungi in the lesion or in the hairs.

Tinea. Ringworm may be caused by one of several species of fungi as outlined above. It is common in children. Tinea capitis produces loss of hair, with broken hairs, "black heads" and dandruff. Tinea circinata is found on the smooth skin of face, neck and body and is a typical "ringworm." The lesions tend to heal at the center and progress peripherally, thus giving the ringlike appearance resulting in the common name (see Fig. 184). Tinea sycosis involves the beard. There are many forms of tinea, all due to closely related fungi.

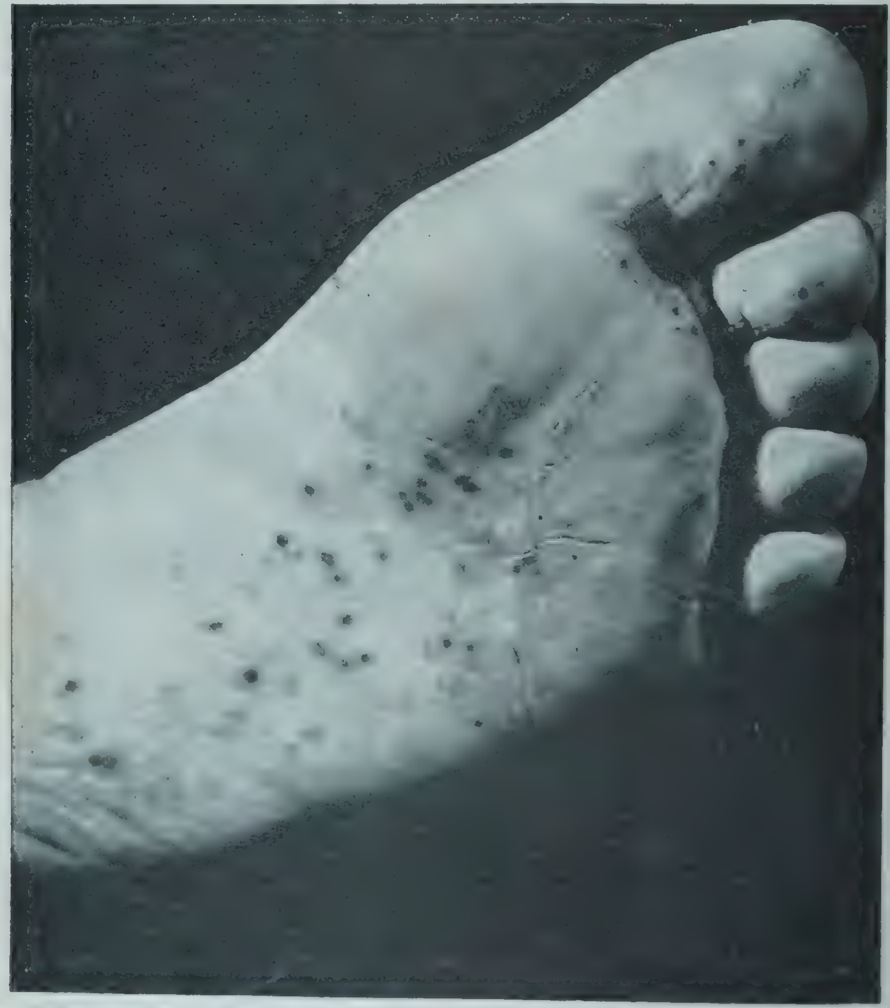

Fig. 186. Moniliasis of foot. (From Swartz, "Elements of Medical Mycology," Grune & Stratton, publishers.)

"Athlete's Foot." This is a term covering various mycotic infections when the lesions are especially manifest in the feet. Infection may occur as a result of walking barefoot in crowded gymnasia, bathing houses, etc., where the floors are warm and wet and the organisms can survive and be readily transferred from one person to another. Various causative agents of the *Microsporum* and *Trichophyton* groups are involved. Cleanliness of feet and care in avoiding infected places with bare feet, will aid in preventing mycotic infections of the feet. As the same organisms also invade the skin of the crotch or groin and adjacent areas, producing tinea cruris, care in using clean, disinfected clothing, suspensories, and towels is desirable in all persons, especially athletes. Persons who perspire much are more likely to contract mycotic dermatitis than are others. The filaments of the organisms are often visible in scrapings from the skin lesions treated with 20 per cent NaOH (Fig. 185). Other forms of "athlete's foot" may be caused by monilia (Fig. 186).

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Chapter 23

THE "SLIME BACTERIA" (ORDER MYXOBACTERIALES)

By far the most complex and interesting colonial or communal existence encountered in the class Schizomycetes is exhibited by the "slime bacteria" or other Myxobacteriales. Most myxobacteria are saprophytes but there are some important exceptions. Most species of myxobacteria normally inhabit the soil and decaying organic matter such as old wood, dead leaves, and dung. A superficial resemblance of some Myxobacteriales to certain of the protozoa (*Mycetozoa,* or "slime animals") is very striking and is coupled with a mechanism for dissemination of the cells, of a complexity unparalleled in any other order of the Schizomycetes, with the possible exception of the *Streptomyces* (see page 464).

Thaxter, one of the first to study the slime bacteria, described them in 1892 as ". . . a bright, orange-colored growth occurring upon decaying wood, fungi and similar substances, which, although in gross appearance it seemed somewhat highly organized, was found, when examined in a presumably mature condition, to consist of apparently amorphous material without signs of hyphae or spores of any kind."[1]

Life History of Myxobacteriales. The life history of many slime bacteria is divided into two stages or periods. The first ("swarm stage") is a period of active multiplication of the fusiform, oval or rod-shaped bacterial cells; the second is a period in which they become quiescent ("encystment stage") and enclosed in cysts of dried slime in which they are disseminated by wind and water.

The Swarm Stage. Starting with a single cell or small groups of, say, two or three cells, we find them multiplying actively by fission and secreting a slimy matrix in which they all live together in the so-called "swarm stage." Each cell is reddish in color, but the exact nature of the pigment is unknown. The cells are usually tapered rods, reaching a length of from 10 to 15 microns and a diameter of 0.5 micron. There is a definite cell wall. They contain granular masses of undetermined characters (Figs. 187 and 188, *A*). Some observers believe a primitive nucleus to be present but this is doubted by others. Oil droplets and volutin granules have been observed in some species. Others describe species with entirely homogeneous internal structure. The slime material may be beautifully stained by some methods.[2] The rods differ from ordinary bacilli in being highly flexible, often bending themselves into loops and in this respect resembling the Spirochaetales. The cells move forward also, by means of a slow, gliding motion.[3, 4]

In one species, highly pathogenic for fish and of great economic importance (*Cytophaga columnaris*), one cell may be seen to glide over others, stop, reverse, change direction, and so on. The rate of progress varies around 60 μ per minute. Rotary and waving movements, in a fixed position, like a charmed cobra, are also described.

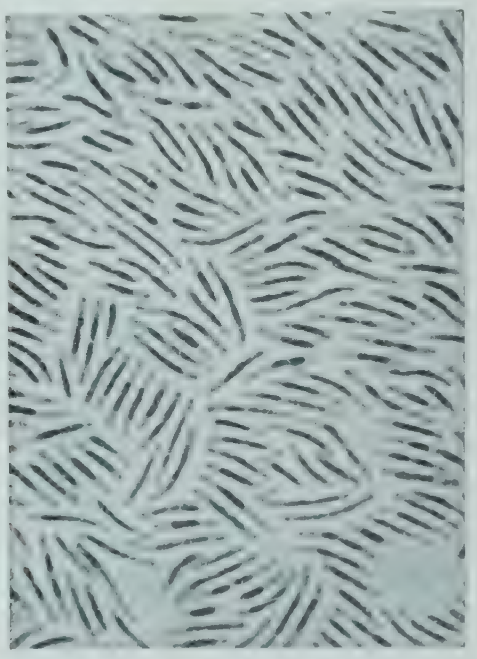

Fig. 187. A species of Myxobacteriales (*Myxococcus xanthus*). Vegetative cells, showing vegetative reproduction. Gentian violet-iodine; × 1860. (Beebe, J. Bact., vol. 42.)

No flagella are seen and none have been described in the entire order.[2, 5]

All the cells in a colony move forward together, secreting slime as they go. On agar plates with young growth "the cells can be seen moving in small parallel groups or singly, away from the center of the colony (which is very thin and flat) and in all directions along curved, clockwise paths."[6]

"A distinct, firm, hyaline, gelatinous base is secreted by the colony as it extends itself, over which the individuals may move or in which they may become imbedded, and is so coherent a structure that whole colonies may be stripped intact by means of it, from the surface of nutrient agar, for example." "In all cases the individuals of a colony are heaped together in the region of its advancing margin which is distinctly elevated above its surroundings, and characteristically roughened by great numbers of partly free individuals projecting from its surface" (Thaxter). This vegetative swarm stage lasts for periods varying from a day or so to a week. Under favorable growth onditions the entire swarm stage growth may cover an area of several undred cm. Such growths have been observed in culture flasks.[7]

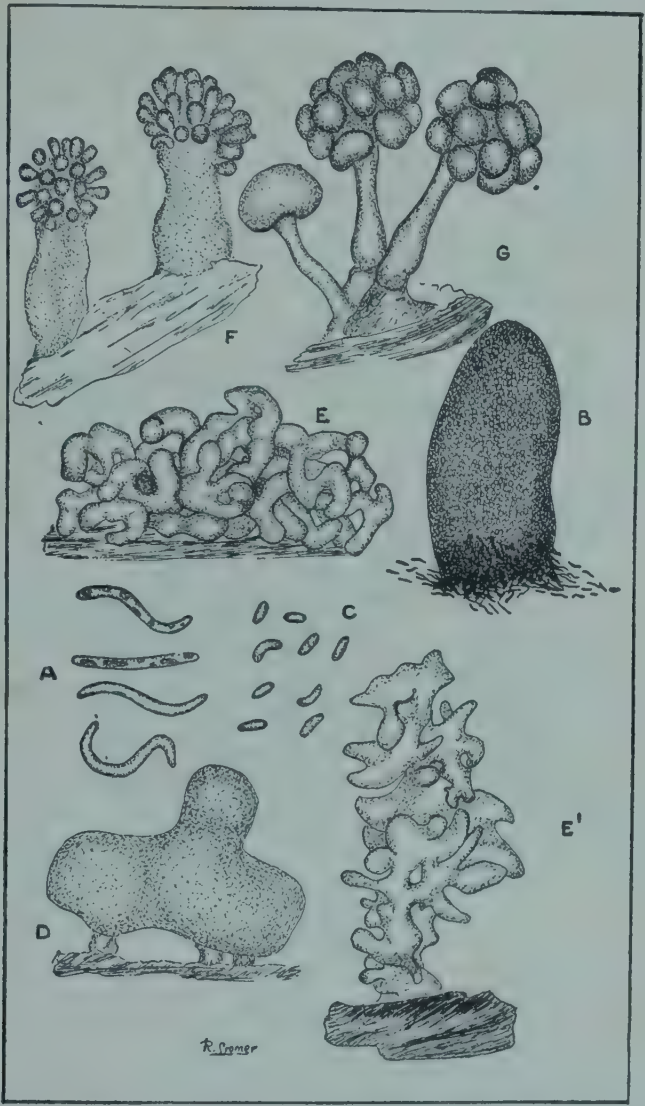

Fig. 188. Various forms of Myxobacteriales. *A,* Vegetative rods; *B,* one form f spore mass rising from rod mass at its base; *C,* short and rounded rods from mature cyst; *D,* low, saclike form of fruiting body (Archangiaceae); *E* and ′, mature cysts of *Archangium serpens* and one other type; *F* and *G,* cystohores and budding cysts (probably Polyangiaceae). (Redrawn from Thaxter.)

The Encystment Stage. Then begins the period of fructification or ncystment. The rods begin to gather together at different points in the margin of the slimy matrix and to heap themselves up. The heaping up may be no more than a low, rounded bulge or ridge. In some forms (Fig. 188, *B*) encystment proceeds directly from this heaping up of cells. The heaps may become raised above the substratum, as

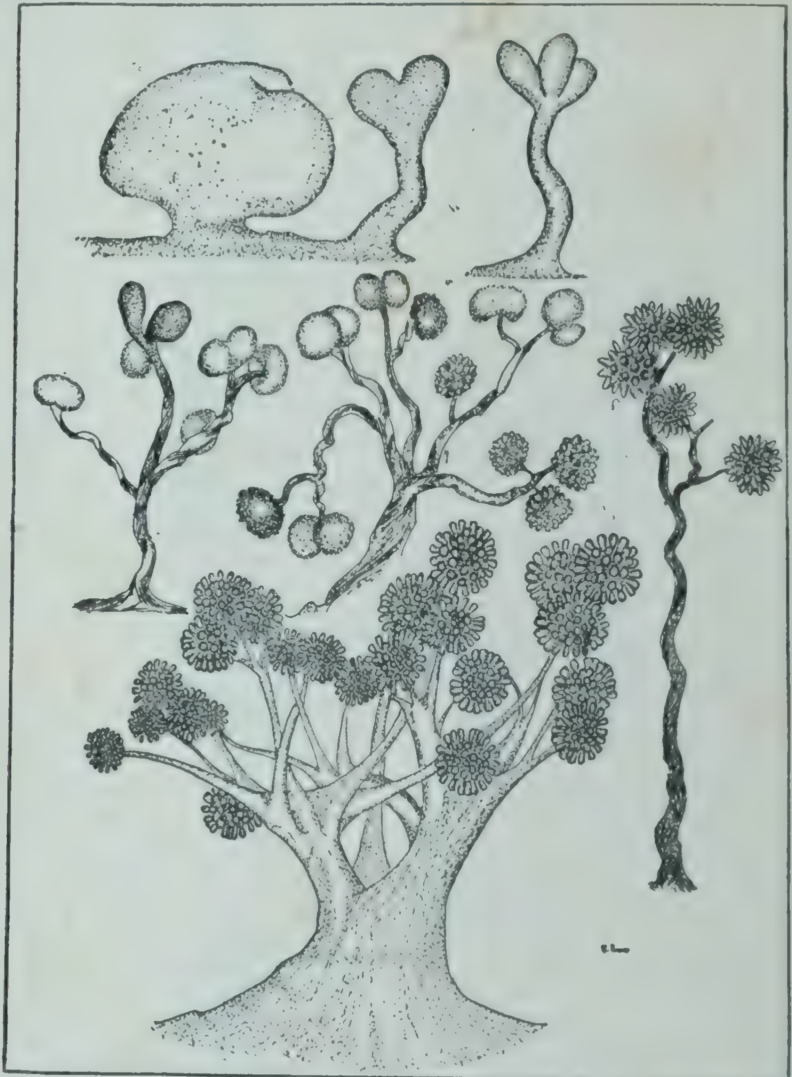

Fig. 189. Various mature forms of Myxobacteriales. (Redrawn from Thaxter.

elaborate projections (Fig. 188, *F* and *G*), the rods pushing to th uppermost portion. The rods become shortened and rounded and ar sometimes spoken of as sporoids. They are not very heat-resistan The gelatinous slime dries as an envelope about them, enclosing the in a cyst or "fruiting body" within which they resist drying and oth unfavorable conditions. These fruiting bodies may be as large as 0. mm. (100 μ) in diameter.

Various degrees of complexity in the heaping-up and cyst-forming process are observed. In one of the most complex and beautiful species (*Chondromyces crocatus*), the masses raised above the substratum continue to pile up, the base of the rising mass becoming constricted and modified into a slender, tubular stalk, often spirally twisted, as much as 1 mm. in height, and of a bright yellow color. Within this stalk of slime the cells proceed upward. The stalk becomes branched, often quite elaborately, and on the tips of these branches the cysts are formed by further subdivisions as ovoid "fruits" (Fig. 189). The rods continue to migrate into the cysts, which then dry and are abstricted from the tip of the *cystophore* and disseminated by wind and rain. The cysts are often brightly colored when growing—red, yellow or orange—and, in large groups, give a very striking appearance to the organisms. After a resting stage, the cysts soften and the vegetative rods emerge as new swarm stages, leaving the empty shell.

Classification of the Myxobacteriales. When the rods enter the cyst stage, they tend to become much shortened and rounded (Fig. 188, *C*). In one group, the cells become so short and round that they resemble cocci. This group comprises the family of Myxococcaceae. Four other families are listed, namely, the Archangiaceae, in which the fruiting bodies are formed of low, irregular, coiled masses and finger-like projections or columnar bodies, but are *not stalked* (Fig. 188, *B* and *D*); the Sporangiaceae, also not stalked, but in which *angular* cysts are formed (Fig. 188, *E*), the Polyangiaceae in which the cysts are rounded and may be enclosed in fruiting bodies or borne separately on the tips of *branched stalks* (Fig. 189),[8] and the Cytophagaceae, in which heaping up is minimal or may be wholly absent.[3a] While all of these organisms are exceedingly interesting, there is more extensive information about the cytophagas and myxococci and only these will be discussed in any detail here.

Genus Cytophaga. These organisms inhabit the soil and sea and are of interest because among them are the most active aerobic, cellulose-decomposing bacteria and because they exhibit several other curious phenomena. The cells are from 3 to 12 microns long and 0.5 o 1 micron in diameter (Fig. 190). They have rounded or pointed ips. Spiral forms are frequently seen in stained preparations but authorities believe these result from distortion due to drying and staining. Cytophagas are stained with difficulty, but are gram-negaive. Giemsa's stain followed by tannin solution may be used.

Cytophagas are strict aerobes, growing well at temperatures beween 10° and 30° C., prefer slightly alkaline media (pH 7.5), and are sensitive to acid. A species pathogenic for fish (*C. columnaris*) has been cultivated readily on nutrient agar and in similar liquid media.[2] The colonies of these organisms are stellate or arborescent Figs. 190, 191).

Their motility is of the creeping and flexing types and is exhibited only in contact with some fixed or solid surface. Sometimes they swing by one end from a fixed surface and oscillate like a pendulum.

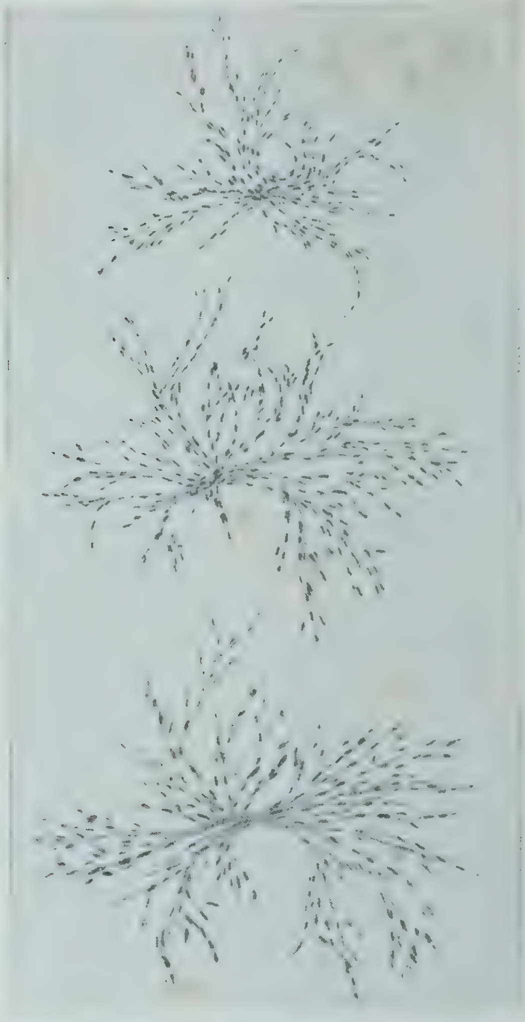

Fig. 190. *Cytophaga columnaris.* Surface peptone agar colonies (72 hours). Semidiagrammatic. ×225. (Garnjobst, L., in J. Bact., vol. 49.)

For cultivation of soil cytophagas an initial enrichment in a solution containing inorganic substances for all except carbon requirement will suffice. Such a solution may consist of

KNO_3	0.1	gm.
K_2HPO_4	0.1	gm.
$MgSO_4$	0.02	gm.
$CaCl_2$	0.01	gm.
$FeCl_3$	0.002	gm.
Water	100.0	ml.

*p*H to 7.5

To this are added some bits of filter paper as a source of carbon. Most of these organisms can utilize cellulose as a source of energy, and, in fact, can utilize few other carbon sources, exceptions being cellobiose and glucose. A few species may attack mannite and xylose. It is of interest to note that the fish pathogen *C. columnaris* does not attack cellulose, nor can it tolerate more than around 0.7 per cent glucose. Gelatin and milk are quickly digested. Organic nitrogen sources are required by this species. Other species of *Cytophaga* (*C. johnsonae*) are active in the digestion of chitin. Such organisms are umerous in many natural environments.[3a]

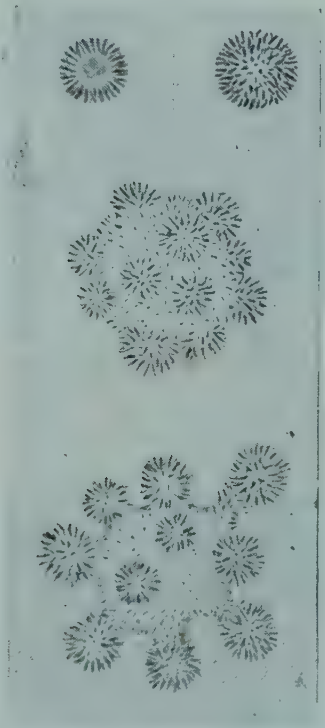

Fig. 191. *Cytophaga columnaris.* Clusters (or stars) formed in 0.5 per cent trypone solution. Living material. Semidiagrammatic. × 125. (Garnjobst, L., in J. Bact., vol. 49.)

As shown by Stanier[3] poured agar plates of this mineral composi-ion may be used with glucose or *finely shredded* filter paper for the ellulose-digesting varieties. A curious fact is that the glucose must be terilized by filtration, since heating, even to 50° C., induces some bscure but very potent unfavorable change to occur in it. Peptone ust be used for nitrogen sources when studying marine forms.

The colonies of soil forms appear at first on cellulose or glucose-ineral agar as yellow, orange or pink discs that have a mucoid ppearance due to the formation of a slimy, carbohydrate, gum-like material. The cells tend to creep outward away from the colony, forming mucoid outgrowths that spread more or less extensively and rapidly, depending on the species. The phenomenon is that re-

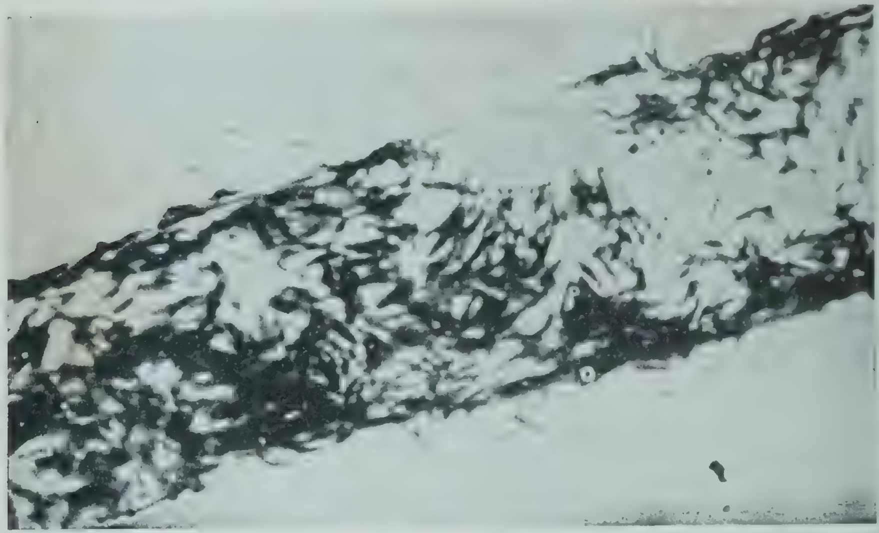

Fig. 192. *C. rubra.* Attacking filter paper fiber. Winogradsky stain. × 835. (R. Y. Stanier, Bacteriological Reviews, Vol. 6, Williams & Wilkins Co.)

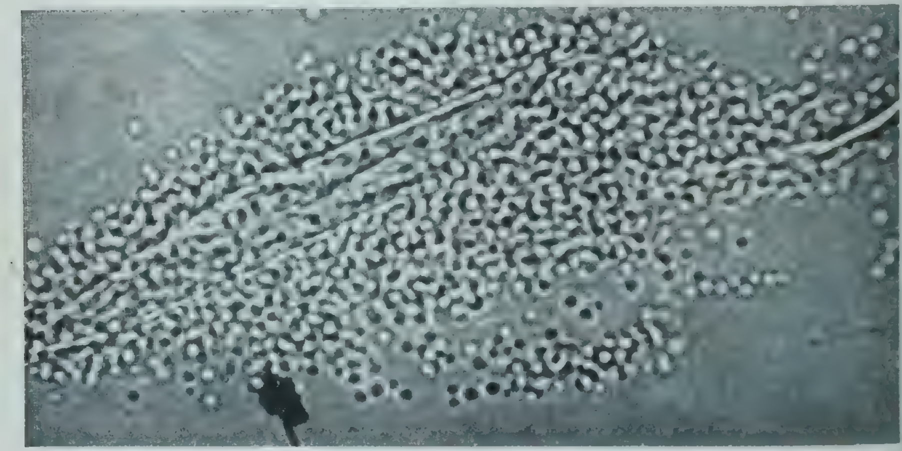

Fig. 193. *Sporocytophaga myxococcoides.* Examples of the typical "Mikrokokkenschleim" resulting from microcyst formation on attacked cellulose fibers. The material was taken from an impure culture on filter paper after five days at 30° C.; photographed in the living state. Magnification × 940 approx. (R. Y. Stanier, Bacteriological Reviews, Vol. 6, Williams & Wilkins Co.)

ferred to above as swarming. It is well exemplified in the order of Myxobacteriales. Swarming is particularly well developed in marine cytophagas. It is interesting that these forms are obligately halophilic, failing to grow well in saline concentrations of less than about 2 per cent. If the seas were the original habitat of living forms, it may be that halophilic marine cytophagas are more primitive creatures than those adapted to waters of much lesser salinity and to soil.

Some species of *Cytophaga* produce dormant bodies called microcysts or sporoids. These develop by a shortening and rounding up of the whole cell. They are surrounded by a mucilaginous sheath (Fig. 193). Microcysts are unlike the endospores of true bacteria in having little greater resistance to heat than the vegetative cell.

The Myxococcaceae. A very interesting property of these organisms in addition to their variable morphology[6] is their ability to multiply upon, and apparently digest and utilize, certain killed, and possibly living, gram-negative bacteria (*Pseudomonas*).[7] They produce a proteolytic enzyme which probably digests the bacteria.

An interesting property of *Myxococcus virescens* is the production of an antibacterial (antibiotic) substance in artificial media. Oxford has shown that this organism may be cultivated on rather simple media:

Material	*Per cent*
Sucrose	0.5
$NaCO_3$	0.2
K_2HPO_4	0.1
KCl	0.5
$MgSO_4.7H_2O$	0.05
$FeSO_4$	0.001
Agar	2.0

or on a fluid medium containing a similar mineral base with the addition of 0.85 per cent acid-hydrolysed casein. No vitamins are needed and certain amino acids are very inhibitory, *e.g.,* glycine, alanine, and cysteine. Growth occurs in shallow media on the bottom of the flasks. The antibacterial substance is active against staphylococci and requires further study.[7]

Cultivation of the Saprophytic Myxobacteriales. The cultivation of most of these organisms is not difficult. One method is to place pellets of sterilized rabbit feces close together on the surface of sifted, fresh soil in covered dishes and to keep the whole quite moist for a week or two at about 35° C. Many species grow well at lower temperatures; from 10° to 20° C. Pure cultures of some species may be obtained on rabbit-dung agar or on infusion agar. Species found growing on otting vegetation or dung are best cultivated on material resembling hat on which they are found. The exact physiological requirements of the organisms are not well known. Peptone seems to be important, but dextrose of little use. Species vary in their acidity requirements; fairly wide range is found in some. Special methods for cultivaon of certain cytophagas and myxococci have been mentioned above. The field is an interesting one for the student of cryptogamic botany.

Relationship to Other Forms of Life. From a practical standpoint most of the Myxobacteriales are of little known importance. From a systematic standpoint, however, there is much of interest. We have already indicated certain points of similarity between the Myxobacteriales on the one hand and the Spirochaetales and certain protozoa on the other, as illustrated in the flexibility and active bending motions of the rods found in the swarm stage of the myxobacteria. The translatory motion of the individual rods of slime bacteria is suggestive of the same type of motion found in sulfur bacteria (*Beggiatoaceae*), in diatoms (*Navicula*), and algae (*Oscillatoria*), while the secretion of slime, a striking character of the Myxobacteriales, is also found in the Cyanophyceae, in true bacteria, in the iron bacteria and in the sulfur bacteria. The remarkable communal existence of the Myxobacteriales, in which may cells aggregate for the common aim of transportation, reproduction, and dissemination, is reminiscent of higher plant or animal life, as examplified in *Volvox* and *Pandorina*, although there is no differentiation of functions among the cells of slime bacteria.

In this connection it is worthwhile to mention a very interesting example of communal motility as seen in certain species of true bacteria of the spore-forming, aerobic genus *Bacillus*. An example is *B. alvei,* described by Shinn.[9] Another of these organisms, *B. rotans*, was described by Roberts.[10] These organisms are of particular interest in view of the superficial resemblance of the motility of their colonies to that of colonies of slime bacteria.

"The outstanding characteristic of the organism *B. rotans* is the motility of young colonies on the surface of nutrient agar entirely free from excess moisture. This mobility expresses itself in two characteristic forms. In certain colonies a clearly visible rotary motion occurs, whereas in other cases the entire colony, consisting of thousands of cells, may exhibit a migratory mobility, moving over the agar at the rate of 0.01 mm. per second." ". . . motility expressed itself first through unorganized, slowly milling masses of cells which finally synchronized their motion and produced either levo- or dextrorotary plates." "In the majority of cases the periphery was broken by the moving cells before a round colony was formed. The release of the cells from the inner portion allowed the colony to move over the aga with a rapid migratory mobility (Fig. 194). Such colonies assumed the shape of a bullet with a round but somewhat pointed anterio portion and a deeply concave posterior portion" (Roberts).[10] One cannot help wondering if this motile colony might not represent a primitive attempt at communal motility which, however, finds greate expression in the higher order of Myxobacteriales.

In the development of a common, slimy colony and tall fruiting stalks with a resistant encysted stage, we find a surprising degree of similarity to those beautiful creatures, the *Mycetozoa** ("slime animals"). Let us briefly consider the latter in order to make the closeness and, incidentally, the superficiality of the resemblance clearer.

The Mycetozoa. A swarm stage is found, which consists of a mass of *living protoplasm* (not inert slime) in which are many *nuclei*. These masses are capable of ameboid motion and, like true amebae, can ingest *solid particles* of food. These are distinctly *animal* characters. The creatures live on rotten logs, etc., and move about in the moisture and shade like amebae. After several days of such existence they cease to move and reproduction begins. The protoplasm sends up *stalks* on the tips of which *sporangia* (spore-bearing cysts) are formed, in a great variety of the most graceful and delicate forms and of the most brilliant colors. Each sporangium contains many spores and each spore contains a single nucleus and is surrounded by a *cellulose* wall. The spores are dispersed by the wind. In water, each spore germinates, forming a naked, ameba-like creature, which later develops a single flagellum. These flagellate forms multiply rapidly by cell division. They then lose their flagella and later *coalesce* to form the *multinucleate swarm stage.*

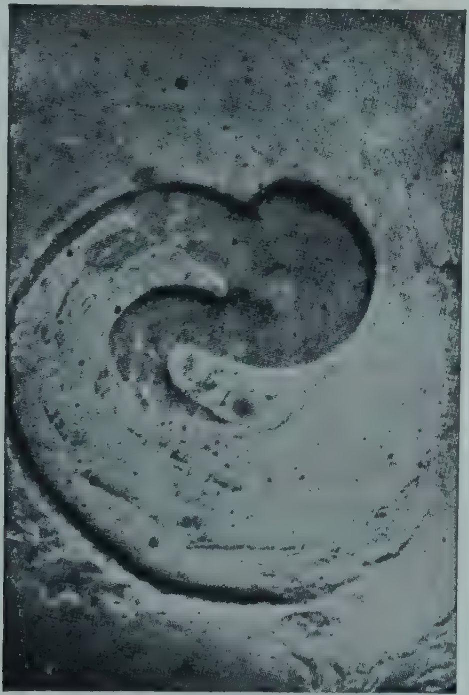

Fig. 194. *Bacillus rotans;* colony on agar. (J. L. Roberts, J. Bact., vol. 29.)

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* Mycetozoa are sometimes incorrectly called Myxomycetes. Myxomycetes is another name for slime bacteria or Myxobacteriales.

Thus, in the formation of a common motile colony, and fruiting stalks, we find strong resemblances between slime bacteria and slime animals. But, whereas the individuals of the *Mycetozoa* pass through a stage definitely animal-like, being motile, flagellate and ameboid, the rods of the myxobacteria always remain definitely bacterial rods. Further, the coalescence of a number of ameba-like definitely *nucleated* slime animals into a *single, living plasmodium* has no parallel in the slime bacteria. The "pseudoplasmodium" of the latter is merely lifeless slime, separate from the cells but in which individual cells live. The entire mass of the plasmodium of the *Mycetozoa* is *living protoplasm.* It is a multinuclear, ameboid cell.[11, 12]

The resemblance between these two forms of life is so close, yet so superficial, as to suggest the idea that a comparative study in morphology and function might have been in progress.

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Chapter 24

THE "SHEATHED BACTERIA" AND "STALK-FORMING BACTERIA"

The term Chlamydobacteriales means sheath-forming bacteria and is derived from the presence of a cylindrical enclosing membrane inside of which the bacteria live and which they may leave behind when they multiply. The sheath is an excretion product, like a capsule or slime layer, but in most of the organisms of this order is a more definite and separate structure than a capsule.

Characteristically Chlamydobacteriales are filamentous with cylindrical sheaths, the sheaths filled with rod-shaped bacteria end to end, like trains of cars in a subway tube. Their habitat is aquatic. They may grow in the sea, rivers, lakes, stagnant ponds and marshes, or in good, sparkling well water, springs, or even filtered water supplies.

Much of our information of Chlamydobacteriales is morphological because few pure cultures have been obtained. True branching does not occur, but in some species one or more cells may be displaced laterally along the course of a sheath and, growing out in a new filament, form a new tube, attached to the first and appearing as a branch. *The sheath is branched* but the individual bacillus-like cells are not. This process is spoken of as "false branching."

We have exact knowledge of the physiology of but a few species, because only these few have been cultivated in pure culture. Most of them may be cultivated in mixed cultures in flasks of their native waters, or on solid objects immersed in their natural habitat. Some are autotrophic, others facultative autotrophs. They are aerobic or microaerophilic. In general, they tend, like many algae, to attach themselves to solid objects, forming slimy surfaces. While resembling some algae in structure and habitat, they are true fungi and are not photosynthetic. They often grow near aquatic green plants since these liberate oxygen into the water. They grow best at temperatures ranging between 15° and 30° C., and in waters or media with a pH around 6.8. Slight acidity, such as that due to dissolved CO_2, favors an increased concentration of ferrous iron (*e.g.,* $FeCO_3$) in the water. This iron favors growth of certain species of Chlamydobacteriales utilizing ferrous compounds as source of energy. Other species prefer situations in which H_2S is being produced by organic decomposition, since these species oxidize the H_2S as a source of energy.

The order of Chlamydobacteriales is divided into three families and some appended groups. The three main families are I, Chlamydobacteriaceae; II, Crenothrichaceae; and III, Beggiatoaceae. An appendix family, Achromatiaceae, includes organisms physiologically like Beggiatoaceae but not filamentous. A second appendix includes organisms less well defined which will not be discussed here.

The first two families include organisms characterized by an extraordinary capacity for abstracting iron from ferrugineous waters and depositing it in their sheath as $Fe(OH)_3$, often in large quantities. For this reason these bacteria have for years been spoken of as "iron bacteria." The Beggiatoaceae and Achromatiaceae, on the other hand, do not collect iron but have for many years been called "sulfur bacteria" because they oxidize H_2S and store granules and globules of sulfur intracellularly in conspicuous masses. Their habitat, metabolism, physiology and structure is related to the occurrence of H_2S in water.

The classification of these organisms exemplifies one of the basic difficulties in taxonomy of the bacteria. In older systems it was customary to include the sheath formers, or iron bacteria, in one order, the sulfur bacteria in another (the Thiobacteriales). In a new system (1948) the physiological property of oxidizing iron is found grouped with the property of oxidizing sulfur, a concept originating with Winogradsky in 1888. The present classification of the order is shown in Table V.

TABLE V.—THE CHLAMYDOBACTERIALES (1948)

ORDER CHLAMYDOBACTERIALES
- Family I. Chlamydobacteriaceae (sheaths)
    - Genus I. *Sphaerotilus* (no iron?)
    - Genus II. *Clonothrix* (deposit Fe in sheath)
    - Genus III. *Leptothrix* (deposit Fe in sheath)
- Family II. Crenothrichaceae (sheaths)
    - Genus I. *Crenothrix* (deposit Fe in sheath)
- Family III. Beggiatoaceae (store S inside cell)
    - Genus I. *Thiothrix*
    - Genus II. *Beggiatoa*
    - Genus III. *Thiospirillopsis*
    - Genus IV. *Thioploca*
- Appendix: Family Achromatiaceae (store S)
    - Genus I. *Achromatium*
    - Genus II. *Thiovulum*
    - Genus III. *Macromonas*

Here we may consider first the "sheath-formers," then the "sulf storers."

THE CHLAMYDOBACTERIACEAE (SHEATH-FORMERS)

Genus Leptothrix. *Leptothrix ochracea,* the best known species in this genus, is world-wide in its distribution. It is found in rivers, lakes, ponds and swamp waters which contain iron in chemical combination with organic matter (*e.g.,* decaying plants).

The yellow- or reddish-colored slime found in the beds of iron-bearing waters nearly always owes its origin to the deposition of ferric hydroxide ("iron rust") in the outer, mucilaginous membranes of the long filaments formed by iron bacteria growing on the stream bottom. Living iron bacteria are, however, seldom found in such iron deposits since the incrustation of the membrane with iron occurs only in maturing filaments and eventually, since it oxidizes to ferric hydroxide and becomes hard and inelastic, interferes with their further reproduction.

The proportion of iron to bacterial substance is computed at about 500:1. This assumes that the organisms are growing as autotrophs, using inorganic ferrous compounds ($FeCO_3$) as a source of energy, oxidizing them to $Fe(OH)_3$. As the yield of energy is only 10 calories per gram of $FeCO_3$, and the organisms are estimated to have an energy utilization efficiency of only about 8 per cent, a very large amount of $FeCO_2$ must be changed to $Fe(OH)_3$ in order to furnish sufficient energy for growth.[1]

According to one view, only those organisms which, as noted above, oxidize *inorganic* ferrous iron compounds, should be classed as true *iron* bacteria. A reaction often given to explain this process is

$$4\ FeCO_3 + O_2 + 6\ H_2O \rightarrow 4\ Fe(OH)_3 + 4\ CO_2 + 40\ \text{cal.}$$

Organisms utilizing only the organic radicle of iron-containing compounds should not, according to Winogradsky, be classified as true iron bacteria. Obviously, those species oxidizing $FeCO_3$ will deposit much waste $Fe(OH)_3$, while those oxidizing only the organic radicles of iron compounds obtain so much energy from the organic matter that relatively small amounts of the ferrous food are needed and little iron is liberated. This iron is probably oxidized by free oxygen extraneous to the cell and yields no energy to the cell.

A difficulty is that there is relatively little *exact* information on the physiology of these organisms.

When observed by ordinary methods, no internal structural details are readily observable in the threads. This has given rise to some difference of opinion as to whether the filaments are coenocytic cells like some mold hyphae, or whether they contain bacillus-like cells which can, when the sheaths become fully encrusted with iron, slip out and acquire a new sheath.

Young filaments of *L. ochracea* may show little or no iron deposition and are of a grayish color and translucent appearance. These forms may be found as grayish flecks or specks in the water of slow streams or stagnant ponds in the spring and fall.

Structure of Leptothrix. The cells are cylindrical and measure 1.5

to 2 microns in diameter by 15 in length. The longer filaments may measure 300 microns in length.

Young filaments may be actively motile, although the presence of cilia has not been demonstrated. The young threads sometimes adhere to the outside of older threads, giving the effect of branching. According to some writers, there is true branching in some species of *Leptothrix*.[2]

Multiplication of Leptothrix. Multiplication may be by one or more of several methods. As noted above, cells may creep out of the old sheath and form a new filament (Fig. 195). Filaments may also multiply by the method of fragmentation. An alternate mode of multiplication is seen when small protuberances, conidia (or buds?) or "swarmers," form on the outside of the thread. These elongate and eventually break off. Each forms a new thread. They sometimes appear in enormous numbers on the outside of *Leptothrix threads*. A thick crust of ferric hydroxide on old threads is obviously incompatible with such a mode of reproduction, so that it occurs only in young threads.

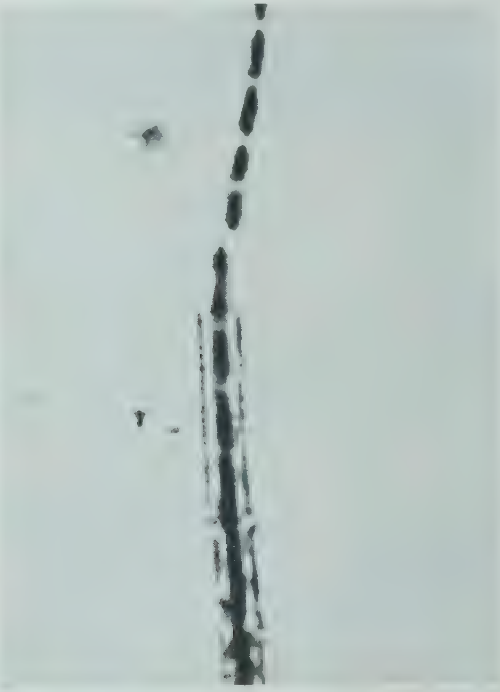

Fig. 195. *Leptothrix ochracea.* Cells coming out of their sheath. About × 2200. (From Cholodny, in Starkey, J. Amer. Water Works Association.)

Genus Sphaerotilus. The genus *Sphaerotilus* is distinguished from other Chlamydobacteriales by the presence, in the bacillary cells within the threads, of large oil droplets and glycogen granules which can be demonstrated by appropriate means (Fig. 196).

The name of a common species, *dichotomus,* is derived from the peculiar habit of forming pseudodichotomous branches. These are best described by quoting Ellis: "Normally the cells at the end of the thread slip out through the open mouth at the top of the sheath, but in addition some cells slip out *laterally,* making their way through the substance of the sheath. Usually these manage to get clear away, but occasionally they remain adherent to the sheath and there elongate to form a thread in attachment to the parent plant. This presents the appearance of a branch fixed to the thread, and when the process is repeated by several other cells in different parts of the thread, and also by the cells of the 'branches,' which therefore in their turn will have 'branches' adherent to them, it can readily be imagined how a colony of threads is formed, as shown in Figure 197."[3]

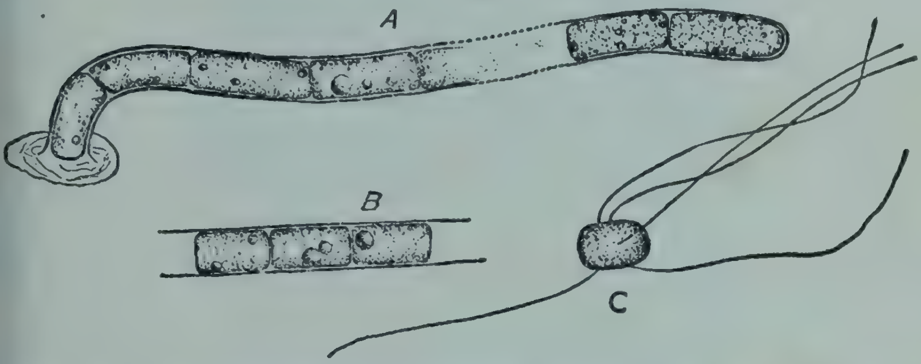

Fig. 196. *A*, Attached strand of *Sphaerotilus.* Note the basal disc and the granular cytoplasm. *B,* Young cells, shorter than old slower growing cells. *C,* Conidium, motile reproductive cell with irregularly attached flagella. (Lackey and Wattie, U.S.P.H.S. Weekly Reports, Vol. 55.)

Structure of Sphaerotilus. Each thread is a tubular sheath, enclosing a single row of cells. *Sphaerotilus* threads remain of a uniform diameter from end to end, since fission occurs in only one plane.* The rod-shaped cells of *Sphaerotilus* are about 2 microns wide and as much as 10 microns in length (Fig. 196). Iron is not deposited in the sheath, or at most in minimal amounts.

Multiplication of Sphaerotilus. Multiplication is by means of fragmentation and by means of motile, ciliated "macroconidia"; the macroconidia are essentially bacterial means of multiplication (fission).

When *Sphaerotilus* multiplies by fragmentation the free fragments of filament are motile by means of cilia, two or three at each end. The lateral escape of cells (macroconidia) through the sheath has already been noted.

* As will be seen in the next section, *Crenothrix* forms trumpet-shaped tubes.

Sphaerotilus Natans. This species is often found in activated sludge (see section on sewage disposal, page 591), and sometimes grows there to such an extent as to clog the tanks, causing a condition technically called "bulking" (Fig. 198).[4]

Genus Crenothrix. The organisms of this genus have a somewhat different structure and mode of reproduction from *Leptothrix* and *Sphaerotilus*.

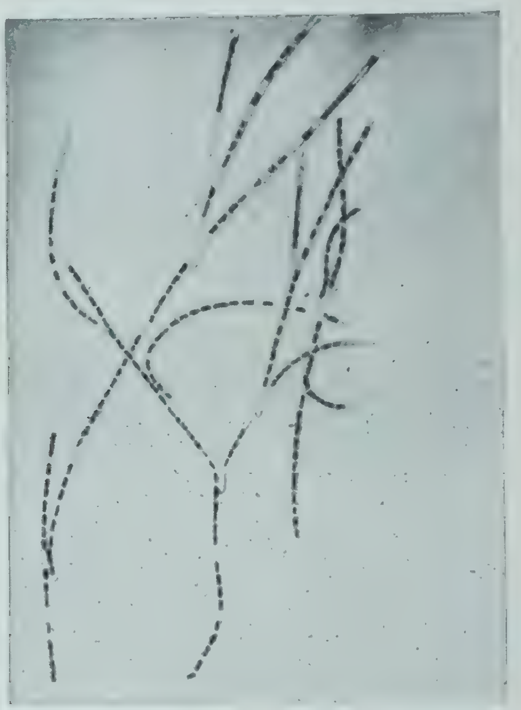

Fig. 197. *Sphaerotilus dichotoma*. Sketch showing false branching. About × 230. (From Molisch, in Starkey, J. Amer. Water Works Association.)

Multiplication of Crenothrix. Crenothrix polyspora, a very common and representative species, starts life as a single, nearly spherical, nonmotile cell about 2 microns in diameter which divides by fission and forms a chain, much as do streptococci, but differing in that the cells elongate as they divide, so that the row shortly consists of bacillary forms. These exude a mucilaginous, tubular sheath of about 0.2 micron in thickness, which keeps the cells in a straight line, and glues one end of the filament to some object. This mucilaginous sheath soon hardens and becomes impregnated with iron hydroxide. The topmost cells are continuously being pushed out of the mouth of the sheath by the growing cells below (Fig. 199). In bodies of water containing much organic matter these filaments are seen as brownish "slime" or hairy covering on rocks.

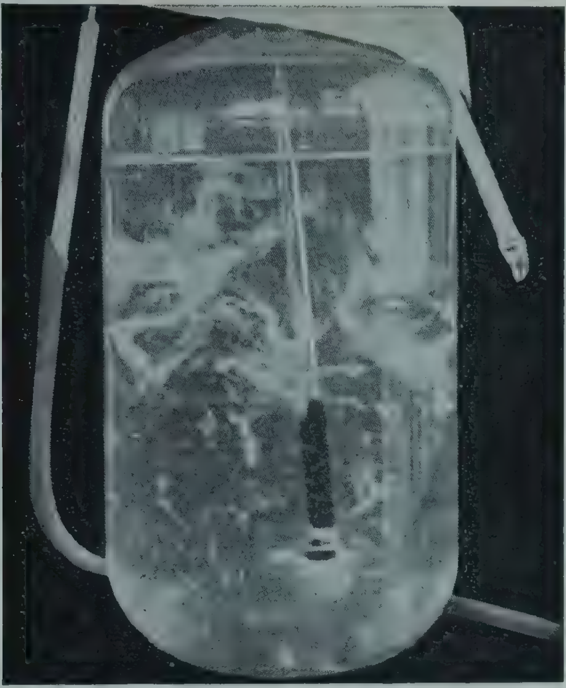

Fig. 198. Free flocs of *Sphaerotilus* in aeration bottle arranged to simulate activated sludge. Air flow is stopped to show the ragged growths which sometimes clog the tanks. (Lackey and Wattie, U.S.P.H.S. Weekly Reports, Vol. 55.)

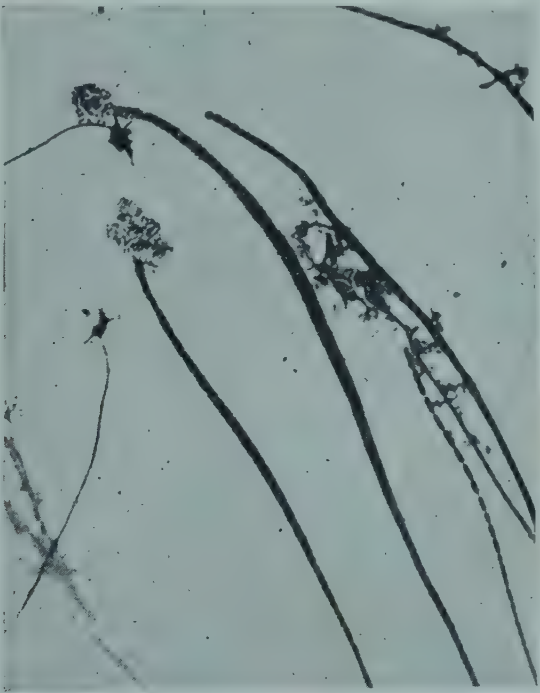

Fig. 199. *Crenothrix polyspora.* Conidia can be seen inside and coming out at the ends of two filaments. × 345. (From Cholodny, in Starkey, J. Amer. Water Works Association.)

The cells thus freed are called *macroconidia* and they start new colonies. Very often, in older plants, fission of the uppermost cells takes place in three planes before their liberation from the sheath. When this occurs the size of the free cells is much diminished and their number increased. Under these circumstances the cells are nearly spherical and are spoken of as *microconidia*. The increase in volume due to the formation of large numbers of microconidia within the sheath forces the latter to expand at the upper end so that a trumpet-like appearance is produced. This is a striking characteristic of *Crenothrix polyspora*.

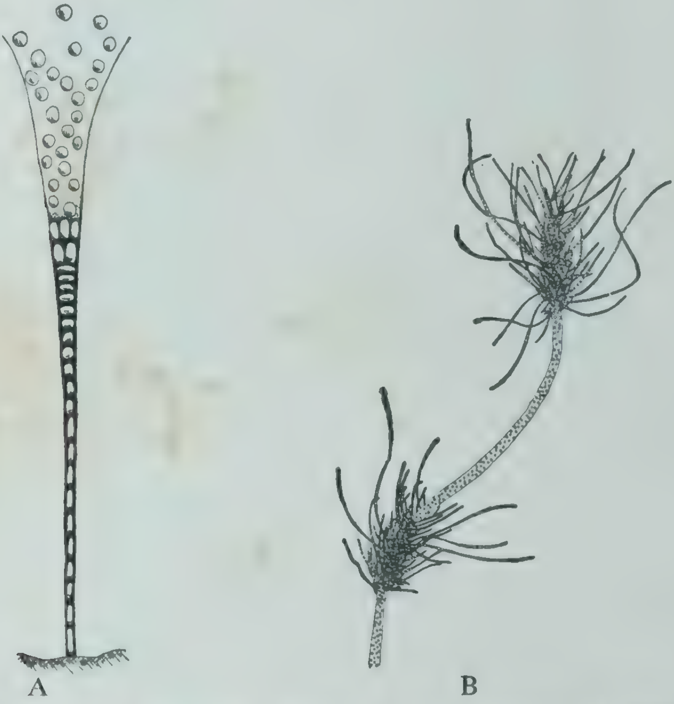

Fig. 199a. *Crenothrix polyspora*, showing, at *A*, method of reproduction by microcysts; *B*, abnormal (?) reproduction. (Redrawn from Ellis.)

Under certain conditions of nutrition, such as an abundance of iron-bearing organic matter,* small numbers of competing organisms and plenty of oxygen and warmth, the cells in the sheath, from top to bottom, *all* start to multiply as microconidia at once and burst through the side, many remaining attached to the exterior of the parent plant and forming new threads, so that dozens or hundreds of brownish streamers may float from one thread (Fig. 199a). Eventually the streamers break off and start the process anew.

Under such conditions of growth *Crenothrix* may, in less than a week, change a large body of drinking water, initially sparkling and clear, to a lake of red-brown fluid which has been aptly referred to as a "horse-pond." The species name of *polyspora* is well merited.

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* Crenothrix is thought to be a facultative autotroph.

Crenothrix can live inside piped water systems, if the water does not contain too much dissolved oxygen and does contain iron. It forms large gelatinous masses that clog the pipes.[5, 6] When the mature plants die under such conditions, they may decompose, with a resultant bad odor and taste that cause discouraging days in the "complaints" office of the head engineers of water purification plants. The saprophytic bacteria which cause decomposition of the dead *Crenothrix* increase in numbers so that the reports of the bacteriological laboratory to the engineer, on the numbers of bacteria present in the water, are also most discouraging, if not alarming. However, from the standpoint of disease causation, *Crenothrix* may be forgotten.

Systematic Relationships of the "Sheath-formers" (Chlamydobacteriaceae and Crenothrichaceae). The relations of the Chlamydobacteriaceae and Crenothrichaceae to other groups of organisms are not entirely clear but in this connection we may consider certain outstanding facts. The order as a whole seems to be more bacterial than moldlike or alga-like. It is entirely aquatic while most molds are not. In the matter of thread formation, the Chlamydobacteriaceae resemble molds, but, as is true of the filaments of the *Actinomycetales*, the threads are much tinier than any mold threads and are of the same order of magnitude as bacteria.

Further, although we speak of the daughter cells resulting from fission within the sheaths as *conidia,* they are really much more like ordinary bacterial daughter cells than the conidia borne on special organs of the molds. If the sheath were absent, they would probably not be called conidia. The absence of demonstrable nuclei is, moreover, a distinctly bacterial character.

Where branching is present, it is seen to be *false* branching due to adhesion of filaments to one another and not to an actual branching of individual cells. The resemblance to molds is again seen to be merely a superficial one.

It may be questioned whether *Leptothrix* consists of coenocytic filaments or bacillus-like cells. However, in *Crenothrix, Sphaerotilus* and *Clonothrix** bacteria-like cells are an outstanding feature. Indeed, cells free from the sheath are hardly distinguishable from cocci or bacilli. The bacillus-like cells frequently are motile by means of flagella.

The Chlamydobacteriaceae and Crenothrichaceae are often classed as "higher bacteria." Actually, if we consider the types represented by *Sphaerotilus* or *Crenothrix* we find them not so very much higher, but, as Ellis says, they may be regarded as bacteria which "have taken the first step toward what may be regarded the communal life."[3] They may represent an approach toward the less highly evolved molds or algae.

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* A genus of organisms much like *Sphaerotilus,* not discussed here in detail.

Role of Iron Bacteria in Geology. A very interesting question in connection with the iron bacteria concerns their part in the formation of iron-bearing geological strata. A number of investigations have been made in this field. We have already seen how the ochre-colored deposits or slimy growths in the bottom of streams and ponds of ferruginous water are often found to be composed of the threads of *Leptothrix ochracea* or some other species of iron bacteria. It is evident that the deposition of iron by such means is slow and that any very large deposits of ore due to bacteria must be so extremely old that the demonstration of bacteria in them would be a matter of great difficulty. In relatively recent deposits, however, such as the cakes of soft iron found beneath peat in bogs ("bog iron ore"), *Leptothrix* and other iron-accumulating bacteria (among them *Gallionella* (see next section) have been found in enormous numbers in various parts of the world. As for older geological formations, iron bacteria or iron fungi have been found in fossiliferous, ferruginous limestones. It is probable, however, that most *large* iron deposits result from purely physical or plutonic actions and are not in any way due to the action of bacteria. Nevertheless it is evident that, tiny though they may be and slow and primitive in their workmanship, these creatures may have contributed to man at least some portion of his "steel age" and to the history of the world.

THE CAULOBACTERIINEAE[7]

The systematic position of these bacteria is somewhat difficult to determine. As will be seen, iron accumulation is a feature of some, but sheath-formation is absent. Formerly accorded the position of a major order (Caulobacteriales) coordinate with the Actinomycetales and Chlamydobacteriales, they are now a suborder in the Eubacteriales.

The majority of the Caulobacteriineae have been known for only a few years or have been given little attention by most bacteriologists. They are of importance in studies of marine and aquatic problems such as ecology of fish and aquatic vegetation, rotting of ships and piling, etc., as demonstrated by studies of Henrici and Johnson,[7] ZoBell, and others. Here we will describe only two species, *Siderocapsa treubii* and *Gallionella ferruginea*. Both of these are implicated in troublesome accumulations of iron in water systems. *S. treubii* is a heterotroph; *G. ferruginea* is a true iron bacterium, *i.e.*, an autotroph using $FeCO_3$ as a source of energy.

These bacteria are almost entirely aquatic or marine in habitat although some have been found in the soil. The living cells themselves are, in general, much like true bacteria in size, shape, lack of visible internal structure, mode of multiplication, nutrition and relation to sunlight. It is logical to include them as a suborder of the Eubacteriales.

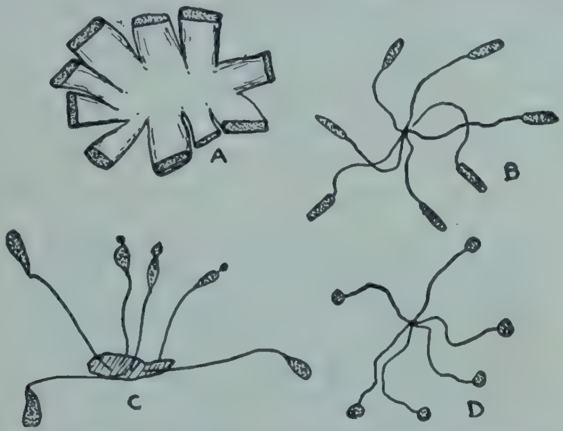

Fig. 200. Various forms of stalked bacteria (Caulobacteriales). *A, Nevskia ramosa,* showing stalks formed by lateral secretion; bacteria (rods) at tips of stalks. *B, Caulobacter flagellatus,* showing stalks formed by terminal secretion. *C* and *D,* other organisms, showing different modes of stalk formation. (Redrawn from Henrici.)

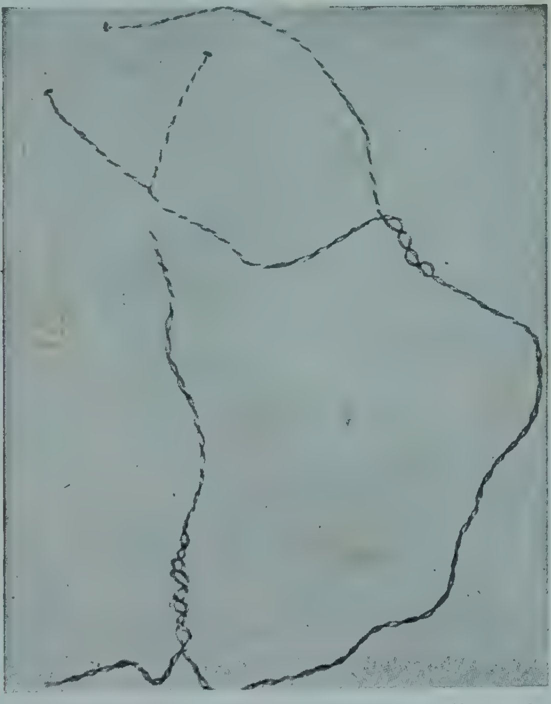

Fig. 201. *Gallionella ferruginea.* Sketch showing cells at the ends of the long, ribbon-like excretion bands. × 540. (From Cholodny, in Starkey, J. Amer. Water Works Association.)

The peculiarity which sets them apart as a separate group is the fact that they form a secretion of gum or other matter *asymmetrically*, *i.e.*, more is secreted at one side or end of the cell than at the other so that, in some species, a stalk is formed as the result of, and consisting of, this secretion. Usually, the stalk is attached to some solid object in the water.

As the cells divide, the stalks become branched so that complex colonies may be formed, consisting of tangles or rosettes of filaments arising from common bases or centers, each stalk having a single living cell at its distal end (Fig. 200). The bacteria do not grow in strings or filaments of cells.

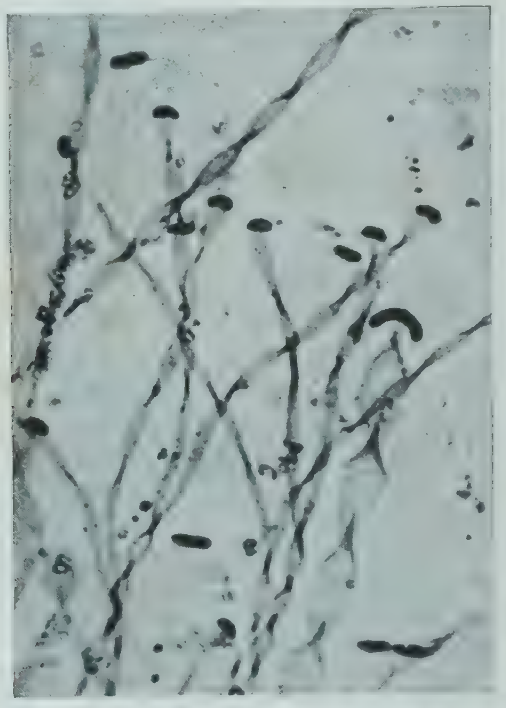

Fig. 202. *Gallionella major*. Curved cells at the ends of excretion bands. × 1120. (From Cholodny, in Starkey, J. Amer. Water Works Association.)

The suborder Caulobacteriineae is divided into four families, the Nevskiaceae, the Gallionellaceae, the Caulobacteriaceae, and the Siderocapsaceae.

Gallionella Ferruginea. This species, formerly included with the Chlamydobacteriales (so-called "iron bacteria") because it forms a stalk of ferric hydroxide, is now classed with the Caulobacteriineae because the organism is neither filamentous nor sheath-forming, but does form a stalk. *Gallionella ferruginea* is the best known species of the order.

The cells of this organism are bean-shaped and about 0.5 by 2 microns in size. As each cell divides branching of the stalk occurs so that complex tangles of long stalks are formed, the stalks sometimes being 0.2 mm. to 0.3 mm. in length (200–300 μ) (Fig. 201).

This organism is found in nature as widely distributed as *Leptothrix*. It can multiply in water pipes, and often causes extensive deposits and incrustations of iron which may eventually occlude the pipes. Problems connected with fouling of pipes, etc., are well summarized by Starkey.[1, 2]

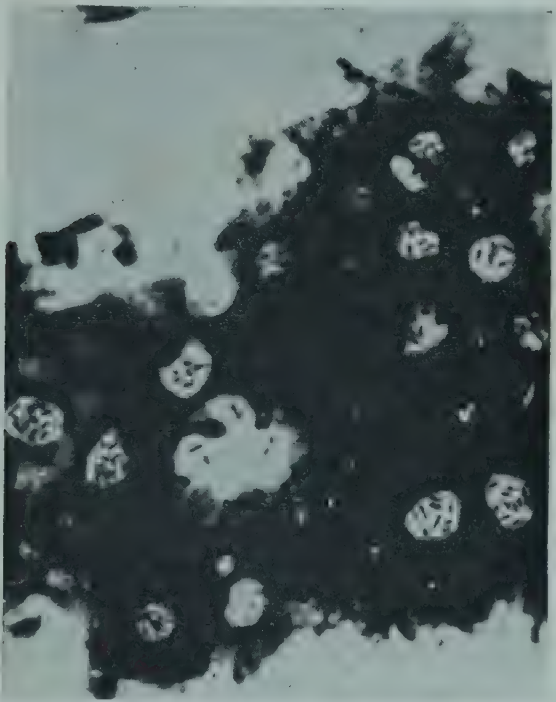

Fig. 203. *Siderocapsa treubii*. Multiple colonies surrounded by ferric hydrate. × 430. (Hardman and Henrici, in Starkey, J. Amer. Water Works Association.)

The stalks of *Gallionella* are flat and have the remarkable habit of twisting so that they resemble a loosely coiled rubber band (Fig. 202). Iron is deposited in this stalk. According to some writers the stalk consists entirely of $Fe(OH)_3$.

The twisting habit renders identification of *Gallionella* easy, since no other organism of similar character is known to twist in just this way. In old plants, the stalk of the organism takes on the appearance of a row of beads due to encrustations of $Fe(OH)_3$.

Siderocapsa treubii is a cocco-bacillus about 0.5 by 2 microns. This organism forms a thick, slimy or gelatinous extracellular substance which does not appear as stalks but in the form of a compact flat mass attached to some object such as a stone, the underside of a leaf floating in a stagnant pond, or a glass slide immersed to collect growths of such organisms in streams. Small slimy colonies or masses of confluent colonies occur, each colony containing 1 to 30 cells and measuring from 2 to 8 microns across. Sometimes these growths are quite extensive and iron is deposited around them. They are probably important in the fouling of pipes but have often been overlooked because their growth is not so extensive as that of filamentous forms. As stated by Hardman and Henrici,[8] "The morphology of this organism as it occurs in its natural habitat is so striking that it can readily be recognized. The iron-encrusted colonies may be readily found with the low-power lens of the microscope." It may grow at a depth of 8 meters. It occurs characteristically in alkaline, hard, lake waters of the drainage type.

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Chapter 25

THE SULFUR BACTERIA AND PHOTOSYNTHETIC BACTERIA

The term Sulfur Bacteria as used here includes organisms which oxidize sulfur compounds or reduce sulfates. Sulfur exists in various stages of oxidation and reduction in amino acids, proteins, and in inorganic compounds ranging from the most reduced, H_2S, through elemental S, thiosulfates, tetrathionates, etc., to the most oxidized form, sulfates. Any of these, except sulfates, may be oxidized as energy sources by various bacteria; and any, except H_2S, may be reduced by still other bacteria. Sulfur and its compounds, therefore, are exceedingly important in bacterial life and, because sulfur in its various forms is essential to plant and animal life, the bacteria that produce the transformations of S, *i.e.,* the so-called sulfur bacteria, are basic to human affairs. In addition to the formation of sulfates which are plant food, sulfur acids are produced by sulfur-oxidizing bacteria, which liberate phosphorus in the soil as soluble phosphates. Phosphates are among the most important and expensive fertilizers. On the other hand, sulfate-reducing bacteria remove acidity of water and soil, thus improving it agriculturally and industrially.

SULFUR REDUCTION AND OXIDATION

Sulfate-reducing bacteria are widely distributed, especially in polluted waters, the sea and marine muds. They have not been very widely studied but are extremely important. One of the best known types is a spirillum-like organism which is classified under the generic name *Desulfovibrio* (Eubacteriineae). These are simple, unbranched, nonfilamentous forms.

Desulfovibrio are strict anaerobes and heterotrophic. Organic materials are dehydrogenated and the hydrogen is transferred to sulfates which are reduced to sulfides. An equation illustrating this reaction is:

$$\underset{\substack{\text{Sodium lactate}\\ \text{(hydrogen donor)}}}{2\ CH_3{\cdot}CHOH{\cdot}COONa} + CaSO_4 \rightarrow \underset{\substack{\text{Sodium}\\ \text{acetate}}}{2\ CH_3{\cdot}COONa} + H_2S + CaCO_3 + CO_2 + H_2O$$

The decreased sulfate content of oil-well brines and other facts, such as ability of sulfate-reducers to transform hydrocarbons, suggests an association between these organisms and the formation of petroleum. These details are more fully discussed by ZoBell in his interesting monograph.[1] There are also sporeforming and other types of sulfate-reducers.

High concentrations of H_2S often occur locally as a result of the activities of these organisms; so much, in fact, as to kill off other flora and fauna. On the other hand, it is in such H_2S-rich locations, *i.e.*, marine muds, polluted waters, sewage, etc., that the sulfur-oxidizing bacteria thrive and help make the region habitable to other forms of life. *Desulfovibrio aestuarii,* a well-known species, can reduce sulfites, thiosulfate and elemental sulfur to H_2S.

The sulfur-oxidizing bacteria may for convenience be divided into three main groups:

The first group comprises the families Chlorobacteriaceae and Thiorhodaceae,* which contain the photosynthetic pigments, bacteriochlorophyll and bacteriopurpurin, respectively. These pigments enable the bacteria containing them to utilize solar energy much as do green plants. These organisms store elemental sulfur in the form of conspicuous granules inside the cells and it is later oxidized to H_2SO_4.

The second group of sulfur oxidizers comprises the families Beggiatoaceae and Achromatiaceae of the Order Chlamydobacteriales (page 432). They do not contain photosynthetic pigment but, like the photosynthetic species, store elemental sulfur *in* the cells. An apt but little used term for all of these sulfur-storing bacteria, both photosynthetic and non-photosynthetic, is "Endothiobacteria."

The third group of sulfur-oxidizing bacteria (the genus *Thiobacillus* of the Order Eubacteriales) has sometimes been termed "ecto-thiobacteria" since sulfur, while used in an elemental form as a source of energy, is not held by them inside the cells but, on being released during the oxidation of H_2S, accumulates as a free scum or precipitate in the medium and is afterward further oxidized to H_2SO_4.

The relations of the sulfur and photosynthetic bacteria are shown in the table on page 447.

THE ENDOTHIOBACTERIA

The bacteria included in this informal but convenient grouping oxidize hydrogen sulfide and other simple sulfur compounds. The photosynthetic species use H_2S as a hydrogen donor in the same manner that green plants use water in photosynthesis. When growing in a H_2S-rich environment the sulfur which is thus freed is stored inside the cells in the form of globules and granules (Fig. 204) where it appears to function as a reserve food substance much as does starch or glycogen in plant and animal cells, and is later oxidized to sulfuric

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* In the Suborder Rhodobacteriineae, Order Eubacteriales.

Relations of the Sulfur and Photosynthetic Bacteria

Reduce Sulfates	Reduce Sulfur, etc.	Oxidize Sulfur		
Desulfovibrio	Various saprophytic species producing H_2S from organic matter: *Pseudomonas*, *Clostridium*, etc.	"Endothiobacteria"		"Ectothiobacteria"
		Photosynthetic*	Not photosynthetic	Not photosynthetic
		Thiorhodaceae	Beggiatoaceae Achromatiaceae	*Thiobacillus*

---

* Other photosynthetic groups *not oxidizing or storing sulfur* are: Athiorhodaceae and Chlorobacteriaceae. The differentiation between Thiorhodaceae and Athiorhodaceae may disappear in pure laboratory cultures since the storage of sulfur granules by the former can be entirely suppressed if they are cultivated in a medium free of H_2S, hydrogen being supplied from other sources. However, in nature, growth of the Thiorhodaceae occurs normally and luxuriantly only where H_2S is found and the sulfur-free growth may be regarded as a laboratory artefact. Sometimes differentiation is very difficult.

acid which immediately forms sulfates. These sulfur granules are a prominent feature of endothiobacteria.

Habitat of Sulfur Oxidizing Bacteria. Sulfur bacteria, both photosynthetic and non-photosynthetic, are found in water and swampy soils all over the world, especially wherever saprophytic organisms are actively decomposing organic matter with the production of hydrogen sulfide, and where *Desulfovibrio,* etc., are reducing sulfates to H_2S. The growths of *sulfate-reducing* organisms and of putrefactive saprophytes are thus seen to be of importance in producing the hydrogen sulfide necessary to the sulfur oxidizing bacteria which in turn are important because they change S and H_2S into H_2SO_4 which is an important plant food. Because of their use of H_2S the sulfur-oxidizing species may be observed in such places as soil, decomposing sea weed, in rock pools, stagnant woodland pools and sewage. The colored varieties (Thiorhodaceae) develop well when exposed to light. In certain sunlit polluted bays they multiply to such an extent as to cause the entire body of water to look red or purple. Certain species of autotrophic Thiorhodaceae grow in hot sulfur springs, and others around sulfur mines. Occasionally certain of the colorless sulfur-storing bacteria (Achromatiaceae and Beggiatoaceae) grow in water supplies, much as do the iron bacteria, and obstruct pipes and are the source of foul odors and tastes due to their decomposition by other bacteria when they die.

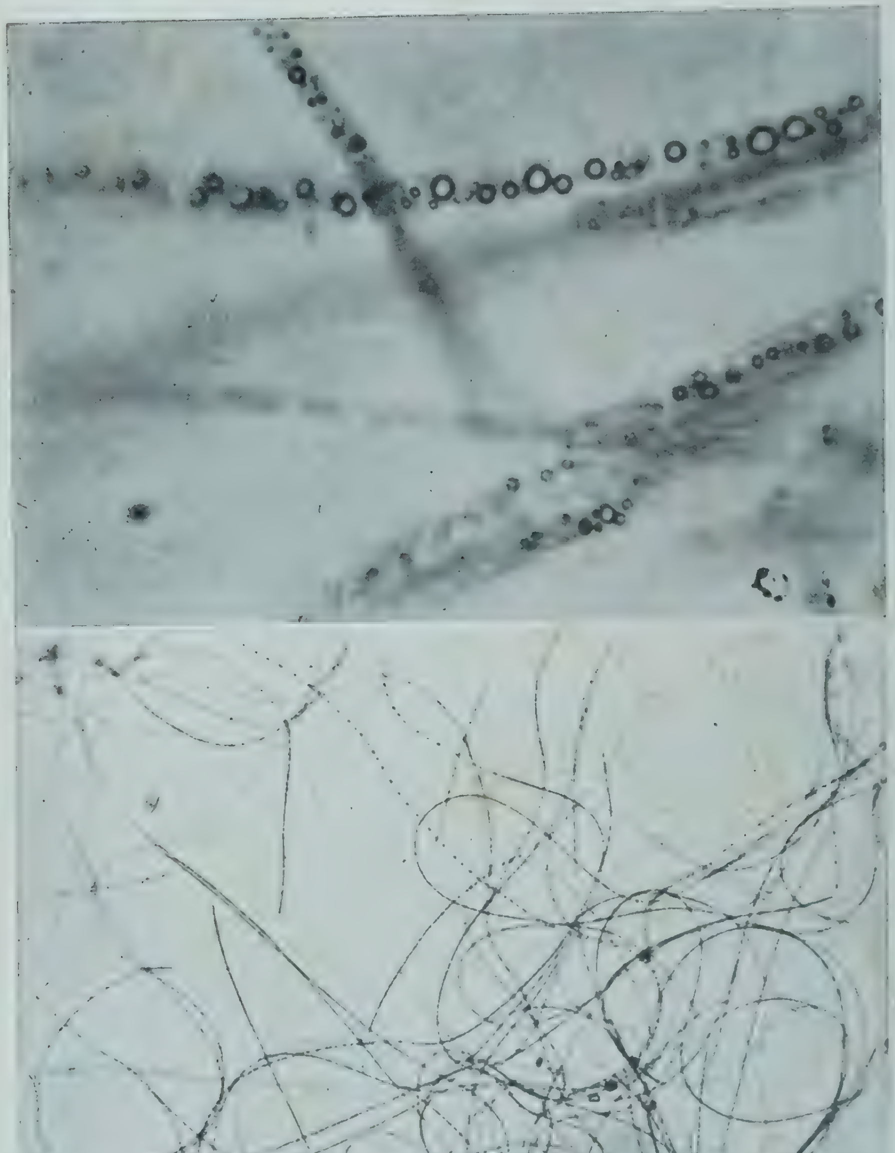


Fig. 204. *Upper*, Portions of actively motile threads of Beggiatoa alba from a picture originally made at a magnification of 2,770. Numerous sulfur vacuoles are distributed throughout the length of the continuous thread. *Lower*, A tangled mass of living Beggiatoa, as seen under a 10 × apochromatic objective and 15 × ocular. (Johnson and Baker, J. Cell. and Comp. Physiol., vol. 30.)

Morphological Characters of Endothiobacteria. As regards morphology, these sulfur bacteria occur in all of the forms found in the Eubacteriales, as well as in filamentous forms. Many of them might be included with true bacteria (Eubacteriineae) were it not for their intracellular storage of sulfur or their photosynthetic pigment. As van Niel states: "If, under certain conditions, one of the non-sulfur purple bacteria (Athiorhodaceae) would fail to produce its prominent pigment system, it would thereby become indistinguishable from a typical *Pseudomonas, Vibrio* or *Spirillum* species."[2] The names of many genera are derived from those of true bacteria by prefixing *Thio* (sulfur); for example, *Thiovibrio, Thiospirillum, Thiococcus, Thiobacillus,* etc.

Nonphotosynthetic Endothiobacteria. The nonphotosynthetic sulfur-storing bacteria utilize hydrogen sulfide and sulfur as sources of energy for the assimilation of carbon dioxide and the synthesis of their cell substance, probably according to the equations

$$2H_2S + O_2 \longrightarrow 2H_2O + 2S$$
$$2S + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4$$

The acid combines with chlorides, metals, etc., to form sulfates. These organisms are autotrophic and aerobic, requiring free oxygen as indicated in the equation given above.

The commonest species of this type is *Beggiatoa alba. Beggiatoa* are filamentous and show creeping and waving movements suggestive of the alga *Oscillatoria.* Typical filaments range in diameter from 1 to 6 microns in diameter and up to several millimeters in length. Another species of the family Beggiatoaceae, *Thiotrix nivea,* forms a sheath about filaments of rod-shaped cells, and is attached to a solid base; properties suggestive of some of the iron bacteria.

Johnson and Baker,[3] by means of light and electron microscope studies, have revealed what appear to be septa or cross walls in young filaments of *Beggiatoa alba,* as well as sulfur granules by sublimation in an *electron* beam and by solution with CS_2. Thin strands, about 0.1 micron in diameter, were seen separated from the outer sheath or cell wall but attached at 1 or 2 ends. These could be confused with flagella. No typical flagella were observed (Fig. 204).

Reproduction of *B. alba* is by fragmentation of the filaments. Fission also occurs as in true bacteria.

Achromatiaceae. Most species of this, the colorless, *nonfilament-forming* family, occur in sulfur springs, sea mud and brine ditches. A good example is seen in *Thiophysa volutans.* This appears *only* as globular or ovoid cells (Fig. 205), never forming filaments. The organisms move slowly in a forward and rotary manner without the aid of demonstrable cilia. The cells are usually large, 9 to 33 microns in diameter, and from 15 to 85 microns long. Multiplication seems to be solely by fission. Various unicellular ovoid, ellipsoidal or round species are known in the four genera of this family, some with cilia, and of varying degrees of complexity of structure and activity.

The Photosynthetic Sulfur and Non-sulfur Bacteria. The photosynthetic bacteria are of two kinds, green (Chlorobacteriaceae) and red-purple (Thiorhodaceae). These utilize hydrogen sulfide, but in an entirely different manner from the non-photosynthetic species. Most are anaerobic (especially Thiorhodaceae). Because of the presence of their photosynthetic pigment these organisms under *anaerobic* conditions obtain the energy necessary for the assimilation of carbon dioxide, from sunlight, *i.e.*, hydrogen is transferred from H_2S to CO_2. Luxuriant growth will not occur in the dark as a rule. When growth does occur in the dark, photosynthesis does not occur and hydrogen is not transferred to carbon dioxide but to *oxygen,* necessitating *aerobic* conditions. The metabolism, in other words, becomes like that of non-photosynthetic aerobic bacteria.

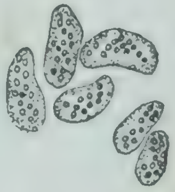


Fig. 205. *Thiophysa volutans* (× 900). Note the sulfur granules. (Redrawn from Ellis.)

The photosynthetic feature seems merely to be added to the ordinary aerobic, oxidative systems of metabolism in these bacteria. The photosynthetic reaction changes hydrogen sulfide to sulfur and represents oxidation. The released sulfur may or may not be stored in the cell as free sulfur granules, depending on the species. Free oxygen is thus not given off in bacterial photosynthesis but free S instead. The red and purple species of Thiorhodaceae retain the free sulfur as intracellular granules which are eventually oxidized to sulfuric acid.

$$2S + 3CO_2 + 5H_2O \longrightarrow 2H_2SO_4 + 3(CH_2O)$$

The Athiorhodaceae, as the name implies, do not store the sulfur but liberate it into the surrounding medium. Both types use hydrogen sulfide in photosynthesis.

The type of reaction developed by van Niel[4] for photosynthesis in general may be adapted to bacterial photosynthesis as a special case in which hydrogen sulfide substitutes for water. In green plant photosynthesis the hydrogen is presumably removed from water under the influence of sunlight and is then incorporated with carbon dioxide in some sort of organic compound generally represented in the reaction formulas as CH_2O. A simplified form of this reaction is as follows:

$$CO_2 + 2H_2O \xrightarrow[\textit{Chlorophyll}]{\text{light}} (CH_2O) + H_2O + O_2$$

The same reaction involving hydrogen sulfide may proceed as follows:

$$CO_2 + 2H_2S \xrightarrow[\substack{\textit{Bacteriochlorophyll}\\ \textit{Bacteriopurpurin}}]{\text{light}} (CH_2O) + H_2O + 2S$$

Many of the photosynthetic bacteria are also able to remove hydrogen from certain organic compounds. This is especially true of the Athiorhodaceae, which are able to use various alcohols for this purpose.[5] Many of these organisms have remarkable nutritive requirements; for example, requiring no amino acids, and no substances of biologic origin, but *1 or 2 vitamins.*[6] The same general reaction holds; hydrogen sulfide is replaced by the organic hydrogen donor in question. For example:

$$CO_2 + C_3H_7COOH + H_2O \xrightarrow[\textit{Bacteriopurpurin}]{\text{light}} 5(CH_2O)$$

Such organisms do not accumulate sulfur granules for obvious reasons. Some of these bacteria can use molecular hydrogen directly. A general formula, representing the type photosynthetic reaction, may be stated as:

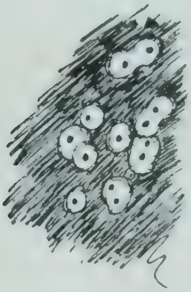
$$CO_2 + 2H_2R \xrightarrow[\textit{Photosynthetic pigment}]{\text{light}} (CH_2O) + H_2O + 2\,R$$

where R indicates the remainder of the molecule from which the hydrogen is abstracted.

Types of Photosynthetic Bacteria. In the family of Rhodobacteriineae the cells may be bacillary, elongated, ellipsoidal, ovoid or coccus-like As noted above, many are exactly like true bacteria except for their photosynthetic pigment. Some, as *Rhodotheca pendens,* secrete quantities of slimy material about themselves, suggestive of some algae (Fig. 206).

An interesting feature of one genus, *Rhodocapsa,* is the presence of *aerosomes.* These are irregular, reddish-colored bodies which refract light and are said to help suspend the cell in water (Fig. 207). Aerosomes also occur in some of the blue-green algae and, together with the slime and the photosynthetic pigment, suggest very strongly that here we may find a transitional stage between these "higher" bacterial forms and the lower forms of algae.

Other thought-stimulating genera of the Rhodobacteriineae are *Amoebobacter* and *Thiodictyon*. In the former the cells are coccoid. They are connected by threads of contractile plasma and exude a slimy, gelatinous matrix enclosing the whole. The threads connecting the cells pull the colony up into heaplike masses, or allow the cells to spread apart (Fig. 208), strongly suggestive of an ameba or of the "swarm" stage of the Myxobacteriales, discussed elsewhere in this section, in which whole families of rods in slime move about from place to place.



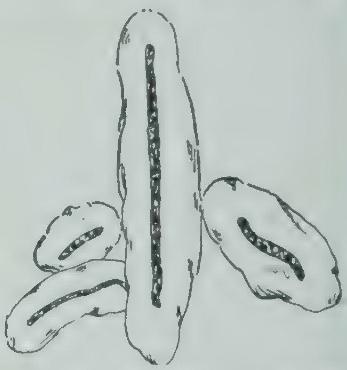
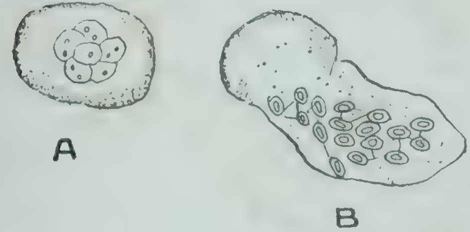


Fig. 206. *Rhodotheca pendens*, showing zoogleal mass (× 900). (Redrawn from Ellis.)

Fig. 207. *Rhodocapsa suspensa*, showing aerosomes and zoogleal material (× 900). (Redrawn from Ellis.)



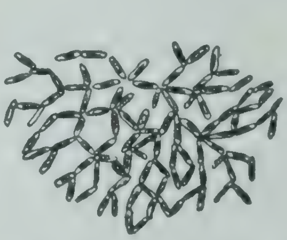


Fig. 208. *Amoebobacter roseus* (× 900). *A*, Contracted; *B*, extended. (Redrawn from Ellis.)

Fig. 209. *Thiodictyon elegans* (× 900). (Redrawn from Ellis.)

Thiodictyon forms a network of spindle-shaped cells united at the ends so that a net is formed which is very reminiscent of the alga *Hydrodictyon* (Fig. 209).

Cultivation of Sulfur Bacteria. The cultivation of sulfur bacteria in bottles of polluted water is not difficult. The photosynthetic types must be exposed to sunlight and air must be excluded. *Pure* cultures have been obtained of certain species of *Beggiatoa* and of some of Rhodobacteriineae, the latter largely through the researches of van Niel. As stated by this author: "It is hoped that the publication of the results may make these bacteria more generally known to microbiologists, and thus lead to move intensive studies of the many fascinating problems which they still present."[2]

"Raw" cultures of sulfur bacteria are obtained in much the same manner as the raw cultures of iron bacteria. The material containing them is *enriched* by the addition of water containing hay, eggs or peptone or, for marine forms, sea water containing decomposing fish or crabs or seaweed. A limited supply of oxygen is desirable and may be obtained by placing the materials in a narrow-mouthed bottle, filled to the neck but left unstoppered. Sea water-peptone agar may yield pure colonies of some sulfur bacteria when inoculated from such primary cultures.

For exact details of pure culture methods the student is referred to the monograph by van Niel, and the article by Foster, since the procedures are rather specialized. For the non-sulfur photosynthetic types (Athiorhodaceae and Chlorobacteriaceae) a satisfactory medium is "a dilute yeast-extract agar containing 3 to 10 ml. liquid yeast autolysate per 100 ml. The addition of a small amount of Na_2S (about 0.01 per cent $Na_2S{\cdot}9H_2O$) and Na_2CO_3 or $NaHCO_3$ (0.2 per cent) is recommended to provide strictly anaerobic conditions from the start, as well as a sufficient supply of CO_2." This is used for shake cultures.[5]

Other Relations of Light to Photosynthetic Bacteria. Some very interesting observations have been made on the effects of light on photosynthetic bacteria. If a spectrum be thrown on a culture of certain Rhodobacteriineae the organisms will congregate most thickly in the infra-red portions and to a lesser extent in blue bands. The infrared is the region in which the bacteria absorb energy for photosynthesis to the greatest extent.* This appears to involve chiefly the green pigment. The red pigment appears to mediate absorption of energy for motion from the blue bands of the spectrum.

THE ECTOTHIOBACTERIA

These are sulfur oxidizing species which do not store sulfur granules inside the cell but liberate free sulfur into the surrounding medium as an intermediary product in the oxidation of H_2S. Two groups are concerned: (1) The family of Athiorhodaceae which are photosynthetic (these have been discussed with the photosynthetic bacteria); (2) the genus *Thiobacillus,* which is discussed in detail here.

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* It is also of interest that the infra-red rays were probably the first to pierce the murky atmosphere of the primitive earth and these bacteria may represent descendants of the first organisms able to make use of photosynthetic processes.

The Genus Thiobacillus. These organisms are classed with the true bacteria and are typical autotrophic, chemosynthetic Eubacteriineae. Thiobacilli are small, gram-negative, nonspore-forming, rod-shaped bacteria, some of which are motile, others nonmotile. They thrive in mud, sea water, and boggy places where sulfur and its compounds are being liberated as a result of protein decomposition, or the activities of *Desulfovibrio* species, or where it exists free, as near sulfur springs. They differ from the endothiobacteria in *not* storing sulfur granules inside their cells as reserve food. On the contrary, the thiobacilli, like the Athiorhodaceae, liberate free sulfur *outside* of the cell.

These organisms oxidize sulfur or its compounds in various ways, two of which are as follows:

1. $5Na_2S_2O_3 + H_2O + 4O_2 \longrightarrow 5Na_2SO_4 + H_2SO_4 + 4S$
   $4S + 6O_2 + 4H_2O \longrightarrow 4H_2SO_4$. (*Thiobacillus thioparus*)
2. $2S + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4$. (*Th. thiooxidans*)

Some species are said to thrive on sulfides, including hydrogen sulfide.

Among the most interesting and completely studied species of this genus are *Thiobacillus thioparus, Th. thiooxidans* and *Th. denitrificans*. These are strict autotrophs. Solutions like the following meet all of their nutritive requirements:

H_2O	100.000 ml.
S	1.000 gm. or
$Na_2S_2O_3$	0.500 gm.
$(NH_4)_2SO_4$	0.030 gm.
KH_2PO_4	0.025 gm.
$CaCl_2$	0.050 gm.
$FeSO_4$	0.001 gm.

Thiobacillus thioparus oxidizes sodium thiosulfate, hydrogen sulfide or sulfur aerobically. The last is transformed quantitatively into sulfuric acid. Imagine giving off sulfuric acid as a waste product! One would become vitriolic without making any voluntary effort.

Thiobacillus denitrificans oxidizes hydrogen sulfide, etc., under strictly anaerobic conditions, using nitrates as hydrogen acceptor and reducing them to nitrites. The metabolism of *Th. denitrificans* is of especial interest since respiration, or oxygen utilization, is often taken by the layman to mean the utilization of free atmospheric oxygen to oxidize organic matter with the liberation of carbon dioxide as a result of respiration. In the case of *Th. denitrificans,* the oxygen is obtained from nitrates, the material oxidized is sulfur or its inorganic compounds, and the product of respiration is sulfuric acid! It is also of interest because this represents one of the factors responsible for losses of fertility in certain soils—*denitrification*, or reduction of nitrates.

Thiobacillus thiooxidans oxidizes sulfur to sulfuric acid aerobically.

As sulfuric acid is formed in considerable amounts, it might be thought that the organisms would quickly inhibit their own further growth. This species, however, is of interest in having a great resistance to acid. It is "distinctive in that it is able not only to tolerate but to produce higher concentrations of acid than any other living organism yet known" (Starkey). Some growth is said to occur at a *p*H of 1, and it grows readily at *p*H3, but it is readily killed by heat (50° C.) and is quite sensitive to drying.

Free sulfur is deposited by most species, as *Th. thioparus,* in granules *outside* the cell proper and forms a scum or precipitate in flasks of medium. All of the other sulfur bacteria, as *Th. thiooxidans,* oxidize it further to sulfuric acid.[7]

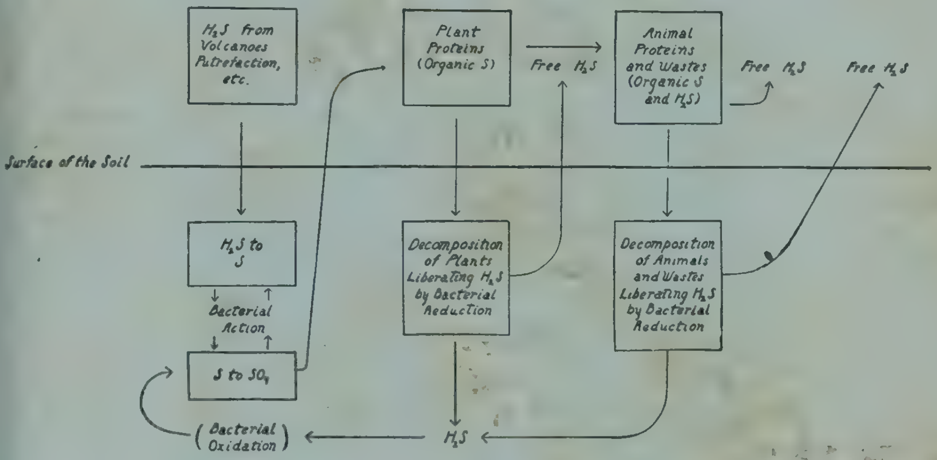
H_2S from Volcanoes Putrefaction, etc.
Plant Proteins (Organic S)
Free H_2S
Animal Proteins and Wastes (Organic S and H_2S)
Free H_2S
Free H_2S
Surface of the Soil
H_2S to S
Bacterial Action
S to SO_4
Decomposition of Plants Liberating H_2S by Bacterial Reduction
Decomposition of Animals and Wastes Liberating H_2S by Bacterial Reduction
(Bacterial Oxidation)
H_2S

Fig. 210. Diagram of the sulfur cycle.

The importance of these sulfuric-acid-forming bacteria as agents in the acid-disintegration of various rocks and minerals, with the liberation of such valuable elements as phosphorus, magnesium, and sodium, must be borne in mind when considering the functions of bacteria in soil fertility. Thus, rock phosphates ($Ca_3(PO_4)_2$) may be transformed to soluble phosphoric acid:

$$Ca_3(PO_4)_2 + 2H_2O + 3H_2SO_4 \longrightarrow 2H_3PO_4 + 3CaSO_4 \cdot 2H_2O.$$

The transformation of barren alkali soils in desert places to fertile ones may be brought about through the activities of such organisms under conditions of irrigation.

On the other hand, such organisms may prove to be important nuisances because acid production in some situations, as in coal mines and iron water pipes, causes costly damage.[8]

Functions of Sulfur Bacteria. The nitrogen of these curious creatures (genus *Thiobacillus*) may be obtained from ammonium phosphate or other inorganic nitrogenous salts not available to higher plants. Carbon dioxide is their source of carbon. They are of importance in making sulfur available for other living creatures and thus furthering the *sulfur cycle*. Sulfur, like nitrogen, is absolutely essential to the formation of protoplasm, and its addition to soil is an agricultural necessity. However, sulfur, like nitrogen, is available to higher plants chiefly in highly oxidized form (SO_4), and the sulfur bacteria are vital sources of sulfates in the soil. A cyclical interchange of sulfur in various stages of oxidation between various living forms is thus seen, and constitutes the basis of a *sulfur cycle* which is, in many respects analogous to the nitrogen cycle (Fig. 210).

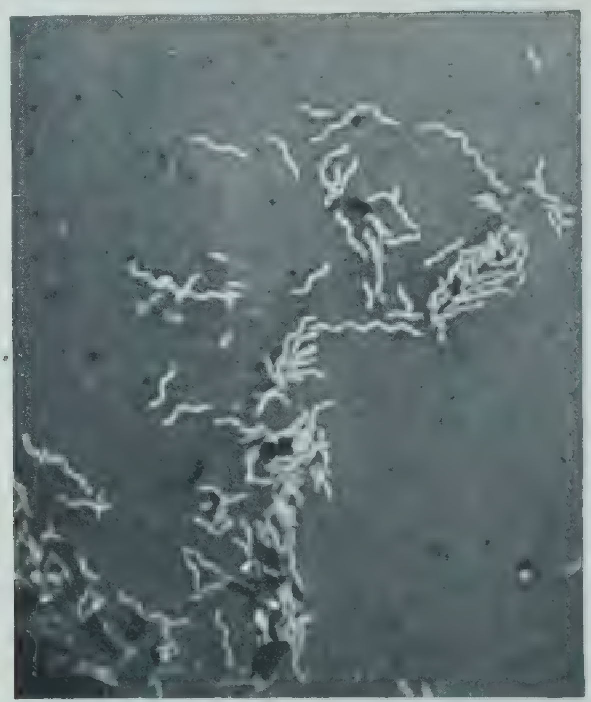

Fig. 211. Spiral cells of the sulfate-reducing *Sporovibrio desulfuricans*. × 1660. (From Starkey, J. Amer. Water Works Association.)

The Sulfur Cycle. Hydrogen sulfide and various oxides of sulfur are given off into the atmosphere by volcanic action and are washed into the soil by rain. Sulfur springs also contain such compounds as well as salts of sulfurous acid, while plant and animal proteins yield hydrogen sulfide upon decomposition. Pure, elemental sulfur is found in deposits in various parts of the world.

The bacteria discussed under the heading *Thiobacillus*, as well as those included in the group of Endothiobacteria and Chlamydobacteriales make use of elemental sulfur, hydrogen sulfide and thiosulfates, as sources of hydrogen or energy, oxidizing these substances eventually to sulfuric acid which combines with carbonates, etc., to form sulfates. In this form sulfur is used by the higher plants and so the bacteria contribute to the support of the human race.

The higher plants and animals build the sulfates into their protoplasm. When they die they are decomposed and most of their sulfur is reduced by the bacteria and other fungi bringing about the decomposition (such species as *Proteus, Clostridium sporogenes, Cl. putrificum*) to free sulfur, hydrogen sulfide and other sulfur compounds like mercaptans, etc. In addition, there are several species of soil and marine bacteria which reduce sulfates with the production of hydrogen sulfide. These sulfate-reducing organisms are included in the genus *Desulfovibrio* (Fig. 211). This power of reducing sulfates is not common in bacteria, and is analogous, with respect to soil fertility, to nitrate reduction. It is essentially anaerobic.

Sulfur in reduced forms is of especial usefulness to the sulfur-oxidizing bacteria in the soil and water, and so the sulfur passes back and forth from the living to the inorganic systems in an alternately reduced and oxidized form. Its initial introduction into the living scheme from its mineral or atmospheric status is largely by means of the sulfur-oxidizing bacteria, while its return to these states is by means of sulfur-reducing species.

The sulfate reducers can grow autotrophically, obtaining energy from the oxidation of hydrogen gas by sulfate which acts as hydrogen acceptor, being reduced to H_2S.[9] These organisms can also oxidize hydrocarbons like kerosene, etc., anaerobically.[10]

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Chapter 26

THE MOLD-LIKE BACTERIA (ORDER ACTINOMYCETALES)

Of the five orders into which the class Schizomycetes is at present divided, the Eubacteriales (true bacteria) is the largest and most widely studied and contains many species of importance in medicine, agriculture and industry. The Eubacteriales are all morphologically simple, unbranched rods, cocci and curved or spiral forms, each individual cell living as a single, independent, and autonomous plant. The greater part of our study (following Part II) will concern these organisms and their role in human woe and welfare.

In this section we will give brief attention to the four other orders of the Schizomycetes. These other orders constitute what are often referred to as the "higher bacteria." Unlike true bacteria, they often form colonies or structures with rudiments of functional differentiation of the parts, and some of them resemble the algae, molds or protozoa in certain biochemical and structural details. The first of these groups of higher bacteria to which we shall turn our attention is the order Actinomycetales.*

This order contains some organisms which resemble true bacteria in size, form, manner of growth, and in existing mainly as simple single cells. However, the cells exhibit slight *branching* under some conditions. This property of branching admits them to the order Actinomycetales. They are the genus *Mycobacterium*. In the same order are organisms which form elaborately branched mycelial structures composed of long, nonseptate filaments, resembling some molds, and which, like molds, reproduce by formation of conidia in long chains. These are the genus *Streptomyces*. Between these two extremes there are groups with various lesser degrees of branching, myclium and conidia formation (Fig. 212).

The definitely mycelium-forming types like *Streptomyces* are regarded by some workers as degenerate molds. Others regard them as derived by evolutionary processes from the simpler Eubacteriales, ranging upward in complexity through the Eubacteriales-like genus *Mycobacterium* to the mold-like *Streptomyces*. Others regard the Actinomycetales as the forerunners of all other forms of bacteria. Whatever their origin, it is evident from what has been said that the

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* The term actinomyces or actinomycetes is sometimes used for the group. Properly speaking, *Actinomyces* is the name of one genus in the group.

Actinomycetales represent a slightly more advanced stage in the evolution of the plant kingdom than do the Eubacteriales.

The name of this order is derived from the term *Actinomyces,* first used by Harz in 1878 to describe the organism (for some time known as *Actinomyces bovis*) causing "lumpy jaw" of cattle. It means "ray fungus" and is descriptive of the peculiar, radial or sunray-like arrangement of the threads of *Actinomyces* which make up granules found in the pus from the lesions of the disease actinomycosis (see Fig. 213). This is not a common character of Actinomycetales, branching cells being the prime differential character of the order. However, *A. bovis* was first of the whole order to be described and is the type species of the genus *Actinomyces.*

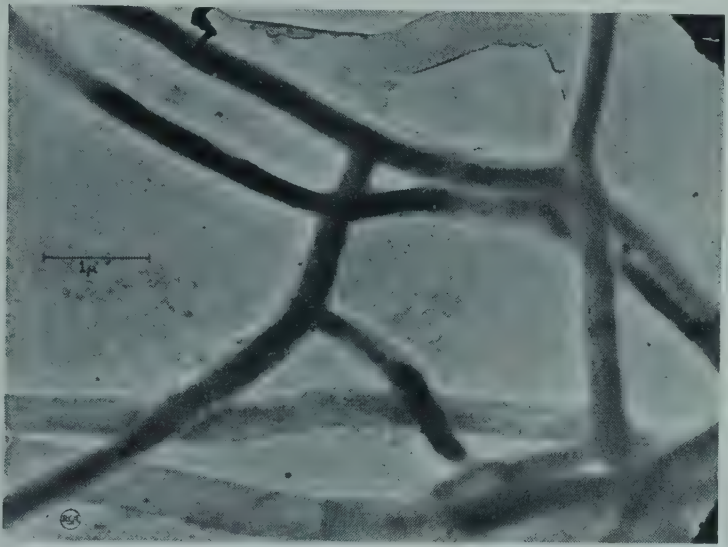

Fig. 212. Electronograph of a species of actinomycete showing branching filaments. The irregular shadows in the filaments are of unknown significance. (Courtesy Dr. Katharine Polevitsky and Dr. R. F. Baker; from unpublished work.)

Those who regard the primitive waters of the earth as the place in which life originated, and the Eubacteriales as the most primitive of known plants, find some support for that view in the numbers of motile, aquatic and marine forms in that order. The Eubacteriales are essentially fluid-inhabiting organisms and many are indigenous to the sea and fresh water bodies. The Actinomycetales, on the contrary, are almost entirely restricted to the soil and are nonmotile, *i.e.,* are not equipped for swimming. On the contrary, most of them are especially equipped for growth on solid surfaces exposed to the air, requiring relatively little moisture in their environment although they will also grow in certain fluids.

Classification of the Actinomycetales. Variability of practically all the properties, such as form and arrangement of mycelium and conidia, pigments, and colony form, which could be used for classifications of the organisms of this group, make the taxonomist's task very difficult. For this reason many different groupings have been proposed, each having its advantages and disadvantages.[1-7] Synthetic media of constant and known compositions, and rather poor in nutriment, induce the appearance of differential characters and tend to stabilize them, while "rich" organic media tend to cause confusing changes. Such media have been used to advantage by Pridham and Gottlieb in studies on the utilization of various carbon compounds as a means of classification of Actinomycetales.[7a]

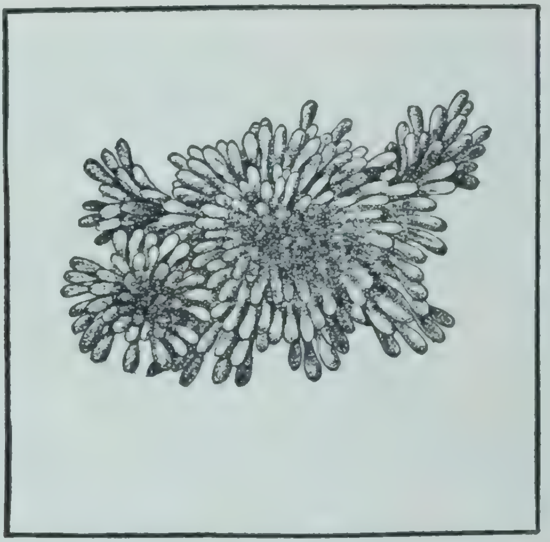

Fig. 213. *A. bovis* from tissues, showing club-formation and radial arrangement giving rise to the name *Actinomyces*. (Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company, publishers.)

A detailed classification will not be attempted here, but certain general segregations which depend on fairly obvious and constant properties can be used to advantage in this discussion.

At present the order is divided into three families. The first (Mycobacteriaceae) contains only the bacterium-like genus *Mycobacterium* which includes the tubercle bacilli and related species.

The second family (Actinomycetaceae) includes true mycelium formers in which the mycelium tends to *fragment* into bacillus-like segments. The anaerobic genus *Actinomyces* of this family includes the pathogenic *Actinomyces bovis* first named by Harz, and a few others which may or may not be different from it. These all tend to fragmentation into bacillus-like segments. A second genus, *Nocardia*, is also included in this family and comprises aerobic filament formers which ordinarily remain unfragmented. None of the members of this family produces conidia.

The third family is called Streptomycetaceae and consists of two genera: *Streptomyces,* which form long, non-fragmenting filaments, and conidia in long chains; and *Micromonospora* which are much like *Streptomyces* but produce only a single conidium at the tip of each fertile hypha. The relationships of these groups are shown below.

The Actinomycetales	
Family Mycobacteriaceae	No Conidia
genus *Mycobacterium* (fragment)	
Family Actinomycetaceae	
genus *Actinomyces* (fragment; anaerobic)	
genus *Nocardia* (do not fragment)	
Family Streptomycetaceae (filament formers do not fragment)	Conidia
genus *Streptomyces*	
genus *Micromonospora*	

General Properties. The principal characters of the Actinomycetales may be tabulated as follows:

Staining
  Gram-positive; some are acidfast (esp. *Mycobacterium*)
Morphology
  Branching rods or filaments; no endospores
Motility
  Non-motile; no flexing or rotation
Catalase
  All produce this enzyme
Colonies
  Tough, irregular, adherent, dry, velvety or "crumbly"
Growth
  Relatively slow (3 days to 2 weeks); improved in 10 per cent CO_2 and by surface-tension reducents
Action on:
  Carbohydrates
    No acid produced as a rule
  Proteins
    Many are actively proteolytic
Nutrient Requirements
  Mostly autotrophic but some require organic C and N. Can metabolize many complex organic compounds as phenol, rubber, paraffin, etc.
Relations to Oxygen
  Aerobic (except *Nocardia,* which may adapt)
Relation to Moisture
  Require little free water

All except the conidia-bearing Streptomycetaceae show a peculiar method of cell fission in which instead of breaking clean apart, the cells often cling together after fission by a shred of cell membrane which causes the two cells to swing around and the lie side-to-side in parallels or bundles. This is often called "post fission movement" or "snapping movements."

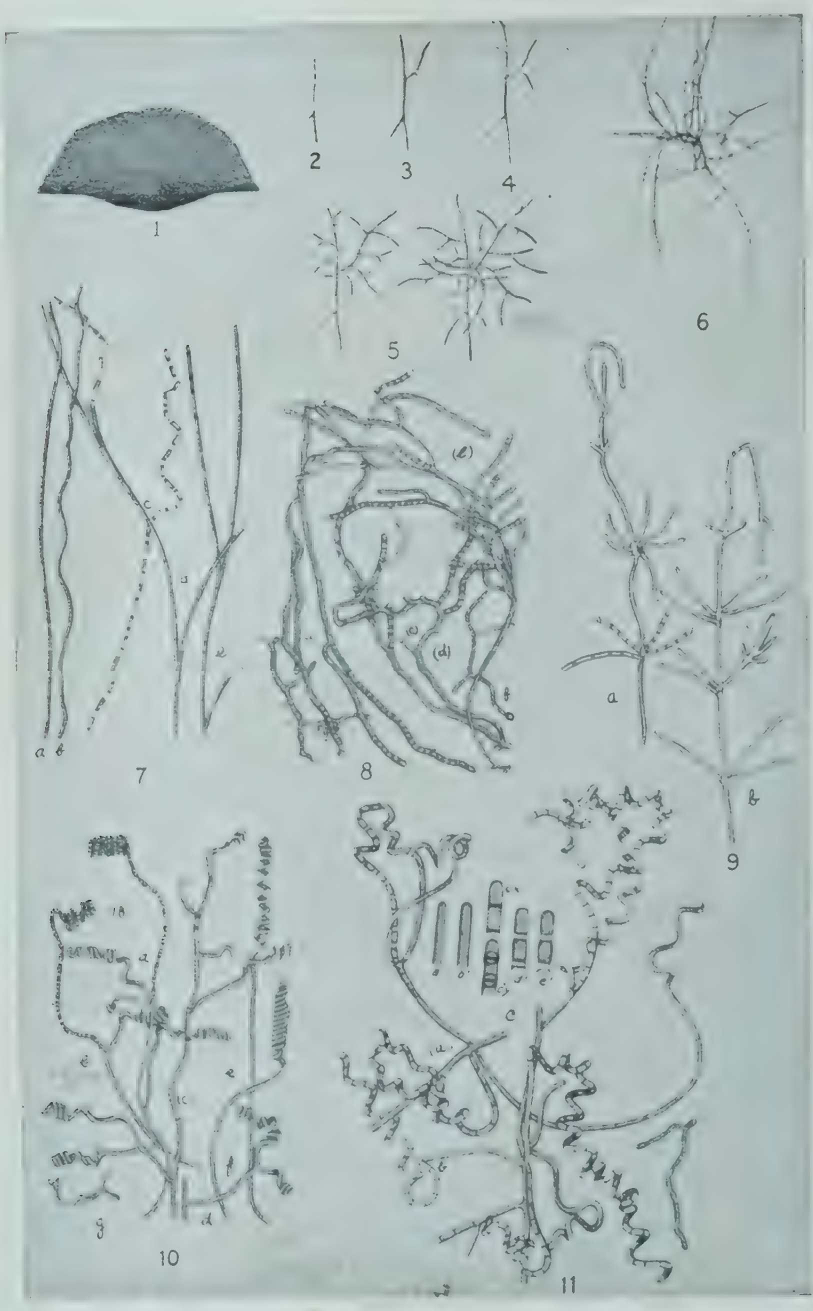

Fig. 214. Details of growth and structure of various Actinomycetales. *1*. Cross section of colony; *2-6*, successive stages in growth from a single spore; *7-11*, various types of mature mycelium, showing different kinds of branching, conidia formation, twisting, fragmentation, etc. (*1-6* from Lieske; *7-11* from Drechsler and Waksman.)

The conidia (often improperly called spores), when formed, are not as resistant as bacterial spores, being killed by ten- to thirty-minute exposures to 60° to 65° C., temperatures only slightly higher than those required to kill the vegetative mycelium. In many species of *Streptomyces* the formation of conidia is accompanied by a corkscrew twisting of the filament so that a very curious, curled appearance is given to the mycelium (Fig. 214).

A striking difference between the moldlike Actinomycetales and the true molds (Eumycetes)* lies in the minuteness of the filaments (1 to 2 microns in diameter) and the absence of visible nuclei in Actinomycetales; distinctly bacterial characters. Granules of volutin and other substances, as well as vacuoles within the older mycelia of Actinomycetales, are often seen and have probably been mistaken by some investigators for nuclei.

There is no differentiation of sexes, such as is seen in molds, and there is neither zygospore nor endospore formation.

FAMILY ACTINOMYCETACEAE

Most of the members of this family are saprophytes, living in the soil as scavengers and attacking complex organic substances of a great variety, such as cellulose, protein, starches, fats, and even carbolic acid (phenol), naphthalene, rubber and cresol as sources of energy or carbon. Their food requirements are otherwise very simple.

Genus Nocardia. Of this genus several species are parasitic in man or animals, one (*Nocardia madurae*) causing "madura foot" or mycetoma in man, others causing tuberculosis-like diseases or ulcerative lesions in man and animals. Most are soil saprophytes.

The colonies of these organisms on agar are often somewhat like those of true bacteria, ranging in diameter from 1 to 8 or 10 microns and being pasty in consistency as a rule. Filaments are inconspicuous or absent, and the organisms in smears often much resemble true bacilli except that branching is sometimes well marked. *Nocardia* may be regarded as intermediate between the mold-like Streptomycetaceae and the bacterium-like Mycobacteriaceae.

All are found in the soil or dust, assisting in the decomposition of dead plant and animal remains, and in their reduction to compounds such as ammonia, hydrogen sulfide, organic acids, alcohols, amines, dextrins, glucose, etc., utilizable by other soil bacteria and by plants.[8]

Genus Actinomyces. These differ markedly from *Nocardia* in being anaerobic or microaerophilic (the only anaerobic organisms in the order Actinomycetales) and in having complex organic food requirements. They are associated with the disease actinomycosis in man and animals. They are much like *Nocardia* in morphology and colony form. They appear to live in the mouth and are not found in the soil like *Nocardia*[7, 9] (Fig. 215). However, *A. bovis* has been found in un-

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* If not already familiar with the general structure of molds, the student is advised to read Chapter 22 on molds.

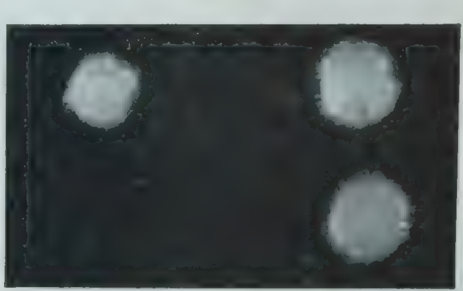

Fig. 215. Typical colonies of Actinomyces. (About 2 ×.) (Bibby and Knighton, J. of Inf. Dis., vol. 69.)

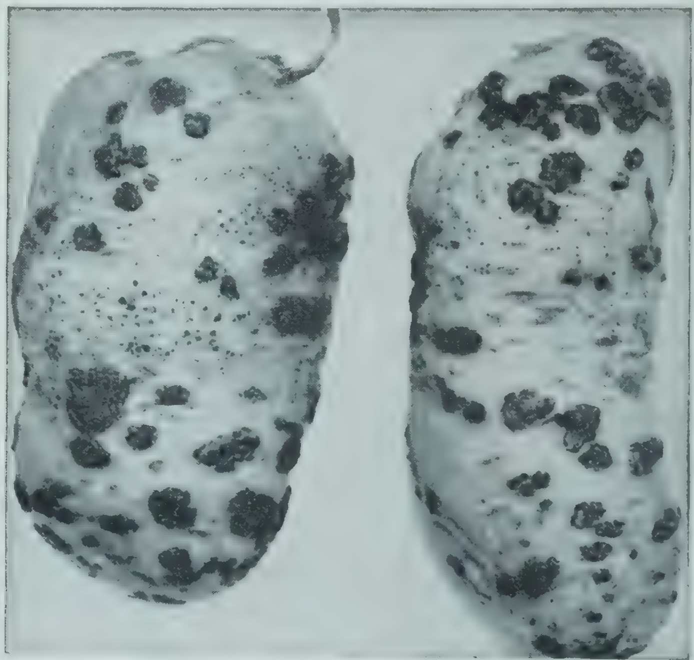

Fig. 216. Potato tubers affected with common scab. (Photo by Bailey, from Owens, "Principles of Plant Pathology," John Wiley & Sons, Inc., publishers.)

opened pustules of fistulous withers and poll evil, two diseases of horses localized in the shoulders and neck of the animals.[9a] Their origin in such lesions is not at all clear.

FAMILY STREPTOMYCETACEAE

This family, comprising the genera *Streptomyces* and *Micromonospora,* includes the most mold-like of all of the Schizomycetes. These organisms are mainly saprophytes and are active in decomposition of all types of organic material. A few are pathogens in animals or plants. *Streptomyces scabies,* for example, produces a troublesome disease (scab) of potatoes (see Fig. 216). Some produce valuable antibiotics; *e.g., S. griseus,* streptomycin.

Cultivation from soil is not difficult, but slow development may permit overgrowth by other, more rapidly multiplying, bacteria if the latter are present in large numbers in the culture. Differentiation of species of *Streptomyces* is often difficult. Conn and Conn have shown that the fact of pigment production *per se,* and the chemical nature of the pigment are more reliable bases of classification than the *colors,* since color in a given species may depend on *p*H of medium and other environmental factors.[6] Cochrane and Conn have recently shown that pigment of one species (*S. coelicolor*) develops when any medium is used which will support *mycelial* growth, provided the reaction remains within the range *p*H 6.0 to 8.5.[10] The best results with soil forms, especially in the development of colors and conidia, are obtained on media such as Czapek's* which, except for a source of carbon, like sucrose, contain very minute amounts of nutrient material, chiefly inorganic.

The flat, tough, colonies on agar are usually from 2 to 5 mm. in diameter, somewhat conical, often papillate, and frequently the surface is thrown into radial folds or ridges or rugae. These colonies resemble very minute mold colonies but are much more compact. They give off a distinctive, musty odor. Some species form more friable, less tenacious colonies. The surface of the colonies (of *Streptomyces* especially) is usually dry-looking and older colonies may be powdery, velvety or woolly, due to the aerial hyphae and conidia.

In order to preserve such material for study, Henrici recommends that agar, inoculated with conidia of the desired species, be spread thinly on sterile slides and incubated in a Petri dish with sterile, water-saturated cotton.[1] After growth has occurred, the slides are immersed in methyl alcohol and stained. This procedure preserves the relations of all parts intact.

Growth is usually best at temperatures around 25° C., although

* *Czapek's solution (modified by Dox and Thom):*

Sucrose	30.0 gm.
$NaNO_3$	2.0 gm.
K_2HPO_4	1.0 gm.
$MgSO_4$	0.5 gm.
KCl	0.5 gm.
$FeSO_4$	0.01 gm.
H_2O	1000.0 ml.

Agar may be added to 1.5 per cent concentration. The reaction should be around *p*H 7.8.

some thermophilic soil species grow well at temperatures as high as 60° C. Growth occurs at *p*H as high as 8 or 9, and is greatly depressed by reactions of around *p*H 5.

It is obvious that liming of acid soils will encourage growth of the saprophytic actinomyces, resulting in increased fertility due to the activities of these organisms in decomposing complex organic materials, including cellulose, so that other bacteria and farm crops can make use of them. The various species causing "scab" of potatoes and other root crops are also encouraged by this procedure so that one must consider the nature of the crop to be raised and soil *p*H before indiscriminate liming. As the Streptomycetaceae and other actinomycetes are aerobic, it is apparent why draining swamp lands increases their fertility.

FAMILY MYCOBACTERIACEAE

This family consists of only the genus *Mycobacterium*. These are definitely acid-fast, gram-positive, nonmotile, aerobic, nonsporeforming rods. Morphologically, they are much like ordinary bacteria except for being somewhat curved and spindle-shaped, and showing beaded or granular cells. The granules are called Much granules after the first writer to describe them and have been regarded variously as regenerative granules, filterable forms and other living forms in a life cycle of the tubercle bacillus. The most acceptable view at present is that they are artifacts, resulting from damage during the staining processes.[14] The idea of a complex life cycle or filterable forms of tubercle bacilli has long been abandoned.[15]

The mycobacteria occasionally branch so that X, Y, L and similar forms are seen. Most of them are harmless saprophytes living in the soil and decaying organic matter. None of them form conidia or aerial hyphae (Fig. 217).

The Ziehl-Neelsen acid-fact stain is of great value in the identification of mycobacteria, since they are unique in being acid-fast.*

Nonpathogenic Species of Mycobacterium. Saprophytic mycobacteria may be found in properly stained smears of material from preputial secretions (*Mycobacterium smegmatis*) or from folds of the skin as in the buttocks or axillae. Saprophytic acid-fast bacilli are also found in butter (*Myco. butyricum*), in manure (*Myco. stercoris*), or on hay (*Myco. phlei*). Many species are also found in the soil where they act as scavengers and bring about organic decomposition. Except for carbon source, their nutrient requirements are relatively simple.

These organisms are readily cultivated at 25° to 30° C. on infusion agar containing glycerin or on coagulated egg or serum. Yellowish or reddish pigments are common. The growth is fairly rapid as a rule (forty-eight to ninety-six hours), a character differentiating these harmless species form the parasitic species *Myco. tuberculosis,* which grows very slowly on such media, appearing only aften ten days to three or four weeks. The colonies of the saprophytic species are usually soft and moist, those of the species pathogenic for man and cattle are dry, rough, crumbly and dull in appearance (Fig. 222). Differentiation among the saprophytic species is difficult and depends largely on a knowledge of the source of any given strain.

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* The filaments of some *Streptomyces* and *Nocardia* are slightly acid-fast and, when fragmented, may closely resemble true mycobacteria.

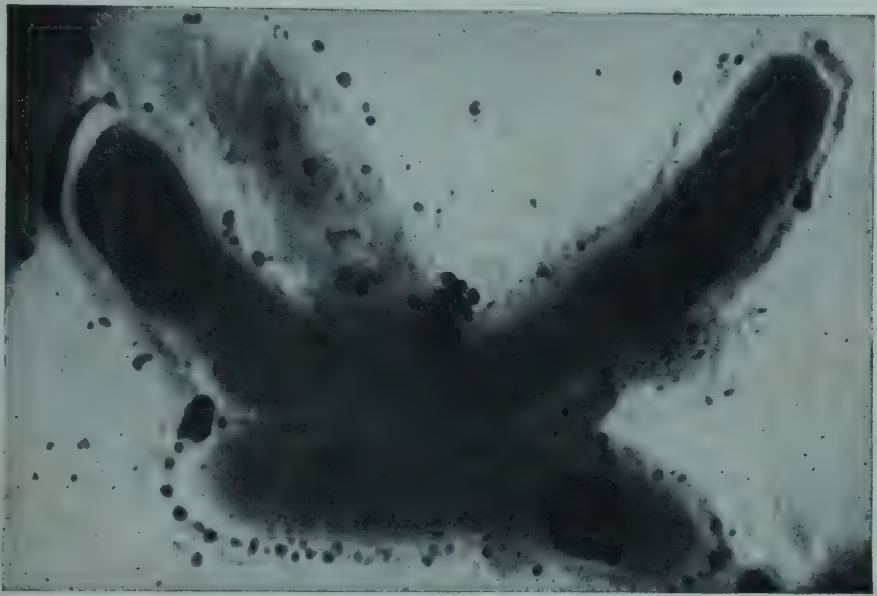

Fig. 217. Electron microscope picture of *Mycobacterium tuberculosis* (× 32,-500). Compare with Figure 30. (Courtesy of R. C. A., Camden, N. J.)

The mycobacteria living in the soil have metabolic powers very similar to those of the soil *Nocardia* and *Streptomyces.* Among their distinctive properties is ability to utilize the hydrocarbons present in petroleum,[16] as well as phenol and cresol. Some species produce pigments with vitamin A properties, synthesizing them from minerals and petroleum.[17]

PATHOGENIC ACTINOMYCETALES

Some *Actinomycetales* are pathogens, although they live in soil as do harmless species.[11]

Actinomyces bovis occurs only in the oral cavity of cattle and other animals and man. Thence it may be introduced into the flesh by thorns, splinters and the like, contaminated with saliva.[7] Swellings are produced and the surrounding tissues become hard and indurated; hence the colloquial term "wooden tongue" for actinomycosis of the tongue in cattle. Eventually the infected tissue becomes riddled with abscess-like cavities which are filled with pus (Figs. 218, 219).

"Madura foot" is a disease of man, common in many tropical countries.* It is similar in many respects to "lumpy jaw" except that the lesions occur in the feet, the organism (*Nocardia madurae*) probably being introduced through cuts or insect bites (Fig. 220). It is confined almost entirely to natives who go barefooted. The Madura foot organisms, of which there are several species, are readily cultivated aerobically on ordinary media.

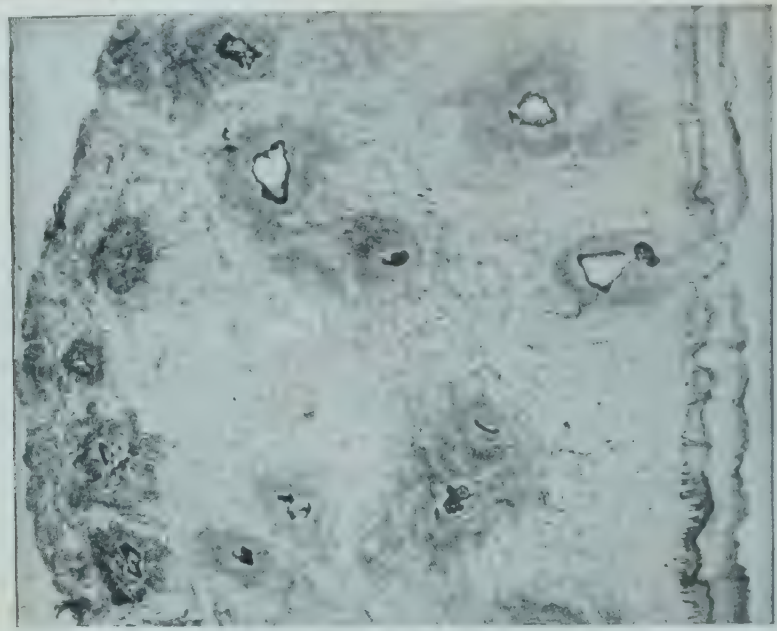

Fig. 218. Transverse section of a part of the lesion of actinomycosis showing numerous abscesses. "Lumpy jaw."

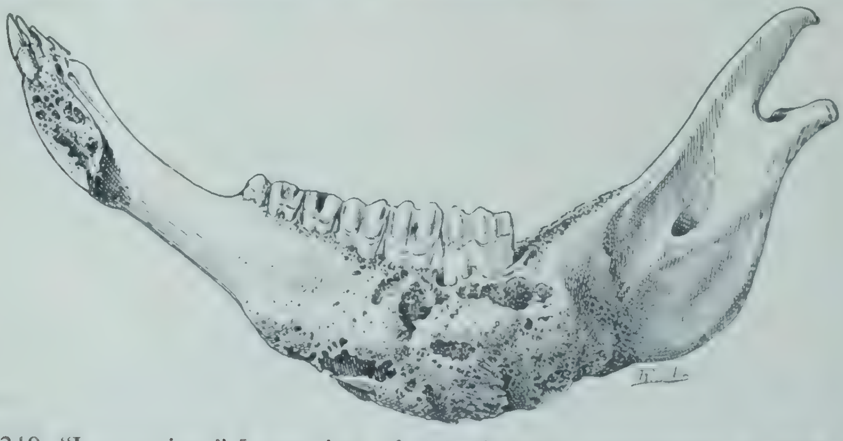

Fig. 219. "Lumpy jaw." Lower jaw of cow showing involvement of bone, with great swelling. (M. Schlegel.)

The organisms concerned in "lumpy jaw" and "Madura foot" tend to form in the pus large (0.5 to 5 mm. diameter) masses of growth with a central core of matted mycelia, the tips of which project at the exterior much like the spines of a sea urchin or a chestnut bur. These tips, unlike a bur or spines, characteristically enlarge and become club-shaped. These masses or granules of mycelial growth,

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* Madura is the name of a town in India where this disease was first described.

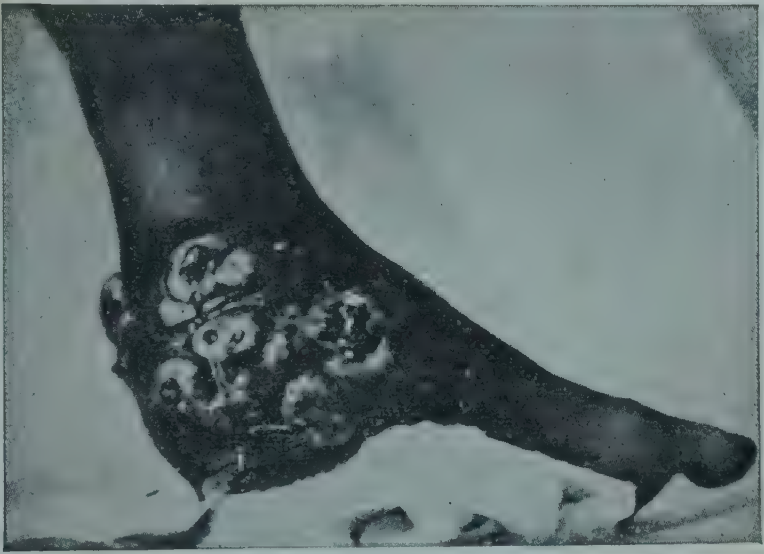

Fig. 220. Madura foot.

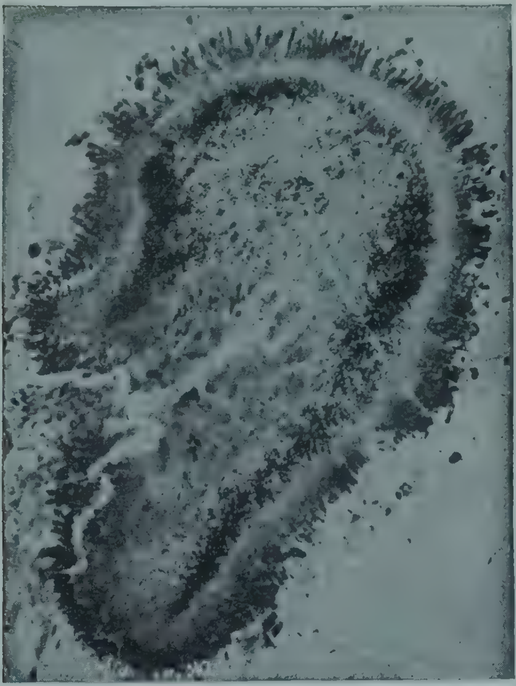

Fig. 221. "Sulfur granule" from a case of actinomycosis. (Wright.) (Photograph by L. S. Brown.)

because of their yellow color, are often called "sulfur granules." Crushed between two glass slides, their mycelial and radial structure is evident and is the basis of the name "Actinomyces" (ray-fungus) first used by Harz in describing the peculiar organism, *A. bovis,* in 1878 (Figs. 213 and 221).

Genus Actinobacillus.* In this place we may mention the genus *Actinobacillus,* although it might more suitably be classified with the Parvobacteriaceae (see Chapter 45).

The genus *Actinobacillus* includes two species, the pathological relationship of which to the *Actinomyces* and *actinomycosis* is obscure but interesting. These species, *Actinobacillus lignieresi* and *A. actinomycetemcomitans,* are small, nonmotile, gram-negative rods. They can grow aerobically on ordinary culture media but growth is much better in serum broth or serum gelatin under an atmosphere containing about 10 per cent carbon dioxide. Although associated with actinomycosis they do not at all resemble any Actinomycetales, and especially they do not resemble *Actinomyces bovis* or *Nocardia* species. They belong with the true bacteria.

TABLE VI

CHARACTERS OF PATHOGENIC ACTINOMYCETALES

Species	Staining Reaction	Relation to Free Oxygen	Pigment
Actinomyces bovis	nonacid-fast gram (+)	microaerophilic or prefers 10% CO_2	cream yellow
Nocardia madurae	nonacid-fast gram (+)	aerobic	orange red
Nocardia asteroides	acid-fast	aerobic	yellow red
Nocardia farcinicus	acid-fast	aerobic	yellow white

Actinobacillus lignieresi causes a disease much like "lumpy jaw," but confined to soft tissue, which is probably often confused with true actinomycosis. "Wooden tongue" of cattle is a common manifestation of the infection. The granules of bacilli found in the pus closely resemble the sulfur granules occurring in the lesions of true actinomycosis. Unlike the granules of true actinomycosis, however, these granules are seen to consist of masses of slender, nonmotile gram-negative bacilli and not of the gram-positive, radial, mycelial network of *Actinomyces bovis. A. lignieresi* can infect man.

Actinobacillus actinomycetemcomitans is often found associated in enormous numbers with *Actinomyces bovis* in the lesions of actinomycosis, but is thought to have no etiological relationship to the disease. It is not pathogenic for laboratory animals. It is found chiefly or only in human beings. In broth cultures it tends to form granules suggestive of sulfur granules.[13]

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* Because of similarity of names, the student must guard against confusing *Actinobacillus* with any of the *Actinomyces.*

Mycobacterium Tuberculosis. This organism, causing tuberculosis is typical of the genus *Mycobacterium*. It is the most strongly acid-fast species of the genus.

Tubercle bacilli do not form spores, but may remain alive for long periods outside the human or animal body. In dried sputum in dark corners they may live six to eight months. In particles of dried and powdered sputum which can float through the air as dust, they can also remain alive for days and may be inhaled. Exposure to sunlight for a few hours kills them and so does pasteurization.

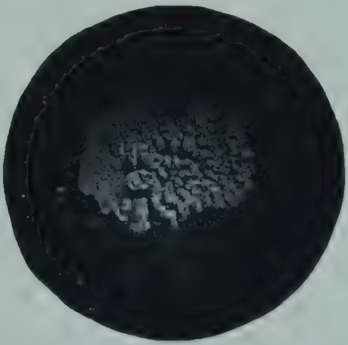

Fig. 222. *Mycobacterium tuberculosis*, human source. Mature colony on glycerol-agar. Actual size. (Swithinbank and Newman.)

Types of Tubercle Bacilli. There are several kinds of tubercle bacilli, varying according to the animal infected. For example, there is a human type (*Myco. tuberculosis,* var. *hominis*) and a bovine type (*Myco. tuberculosis,* var. *bovis*) which grow well at 37° C. A third mammalian type, called the vole* bacillus, was discovered in 1937. This organism is highly virulent for voles but in man produces a mild *immunizing* infection. It is cultivated much as are tubercle bacilli. There is also a bird or avian type (*Myco. avium*) which grows well at about 40° C., and turtle (*Myco. chelonei*), fish (*Myco. marinum*) and other cold-blooded animal types which grow well at lower temperatures (18° to 30° C.).[18] All look alike microscopically and may be cultivated on similar media, although the human type grows well on glycerin media whereas the bovine type does not do so well with glycerin. The cold-blooded animal types do not as a rule infect the warm-blooded animals or birds and vice versa. Human and bovine types seem to infect man and guinea pigs with equal ease but differ in their effect on rabbits, the bovine killing the rabbit within four to six weeks, the human usually not killing at all, or much more slowly (Fig. 222).

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* A vole is a kind of field mouse common in England.

Laboratory Diagnosis of Human Tuberculosis. As is well known, tubercle bacilli often occur in large numbers in the sputum of persons with active pulmonary tuberculosis. For diagnostic purposes the organisms may be demonstrated there or in other material such as urine, pleurisy fluid or feces, depending on the organs affected. The methods available for demonstrating the tubercle bacilli in pathological material are (*a*) microscopical examination of smears of the material, stained by the acid-fast method; (*b*) cultivation on media specially adapted to the growth of the tubercle bacillus and (*c*) by the inoculation of guinea pigs or rabbits, followed by observation of the development of tuberculosis in these animals.[19, 20, 21]

Cultivation of Tubercle Bacilli. When cultivating* from sputum or other contaminated material, it is necessary to kill or inhibit the growth of the other bacteria present, otherwise they will overgrow and kill the tubercle bacilli. The material, especially sputum, is therefore treated with an equal volume of 5 per cent sodium hydroxide for thirty minutes at 37° C. This kills nearly everything but tubercle bacilli and probably kills many of these unless the alkalinity be promptly neutralized. The fluid is neutralized with sulfuric acid and centrifuged, the sediment being transferred to slants of suitable medium. In order to inhibit the growth of any contaminating bacteria resisting the sodium hydroxide, certain dyes are added to the medium. Brilliant green, crystal violet, congo red, etc., will prevent the growth of many bacteria but not of tubercle bacilli. Cultures should also be inoculated with some of the supernatant fluid as this may also contain many bacilli.[21a]

* *Petragnani's medium for Mycobacterium tuberculosis* (*Frobisher's modification*):

Mixture A

Milk	225.0 ml.
Potato starch	9.0 gm.
Peptone	1.5 gm.
Diced potato	150.0 gm.

Heat in double boiler one hour, stirring occasionally.

Mixture B

Eggs	8.0
Glycerin	18.0 ml.
2 per cent aqueous malachite green	15.0 ml.

Mix well.

Mixture C

Dextrose	1.5 gm.
Asparagine	1.5 gm.
Water	50.0 ml.

Warm till dissolved.

Mix A, B and C in a bowl and strain through one layer of gauze. Tube and sterilize in slanting position. Sterilize by inspissation for 1 hour at 80° C. Cotton plug should be saturated with sterile petrolatum to prevent drying out.

Mycobacteria and Wetting Agents. The media traditionally used for cultivation of tubercle bacilli are rich in organic matter and are well represented by Petragnani's medium (see page 472). However, it has long been known that mycobacteria, including tubercle bacilli, have simple nutritive requirements and will grow well in mineral solutions containing 1 or 2 amino acids and the like. Long's synthetic medium has been used for years[22] and Youmans cultivated tubercle bacilli in a solution containing asparagine (0.5 per cent), magnesium citrate (0.25 per cent), glycerol (2.0 per cent), potassium phosphate (0.5 per cent), and magnesium sulfate (0.05 per cent); *p*H 7.0.[23]

A difficulty with such aqueous media is that mycobacteria, especially tubercle bacilli, are rich in wax-like compounds which make them relatively impervious to aqueous fluids. That is, they are not easily *wetted* and it may be supposed that aqueous nutrient solutions do not come into intimate contact with the cell surface. In such media they usually tend to grow in clumps or granular masses and to float like granules of wax on the surface. As a result, under ordinary conditions, nutrition is retarded and growth of the organisms is usually slow, requiring 2 to 3 weeks or more to become abundant, and the use of copious inoculation.

More recently the addition of a wetting agent (surface tension reducent) commercially named "Tween-80"* has permitted the preparation, by Dubos and his colleagues,[24] of rapidly growing subsurface cultures in simple liquid media.† These cultures remain in a

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* Tween-80 (Atlas Powder Co., Wilmington, Del.) is a polyoxyethylene derivative of sorbitol mono-oleate. (Prepare 10 per cent aqueous stock solution in distilled water. Do not use after 1 month. Keep in refrigerator.)

† *Dubos' medium:*

	gm.
KH_2PO_4	1.0
$Na_2HPO_4.12\ H_2O$	6.5
$CaCl_2$	0.0005
$MgSO_4.7\ H_2O$	0.001
$ZnSO_4$	0.0001
$CuSO_4$	0.0001
Ferric ammonium citrate	0.05
Enzymatic digest of casein	2.0
Asparagine	2.0
Tween-80	0.5
H_2O	1000 ml.

Adjust to *p*H 6.8. Dispense in 5 ml. amounts in tubes 25 mm. in diameter. Autoclave.

Then add aseptically to each tube:

Glucose (50 per cent aqueous, autoclaved)—0.05 ml.

Serum albumin fraction V (Armour Laboratories, Chicago, Ill.)—0.05 ml.

Prepare a 5 per cent stock solution in 0.85 per cent NaCl. Neutralize with NaOH. Sterilize by filtration and heat 30 minutes at 56° C. to inactivate lipase which hydrolyzes Tween-80.

The albumin fraction appears to act by binding free oleic acid which is very toxic to tubercle bacilli and occurs as an impurity in Tween-80.[24, 24a]

diffuse state manifested by an even turbidity and no *granule or pellicle formation.* Growth is easily initiated by *minimal inocula.*

Use of Dubos Medium. For primary isolation from sputum digested as described it is recommended that C.P. sodium oleate be substituted for the Tween-80, and that malachite green (1:500,000 final concentration) or 30 units of penicillin per tube be added.[25] Experience with the medium for diagnosis is too limited to evaluate it but it has not given promising results. Dubos did not intend it as a diagnostic medium.

Experimental studies with pure cultures in the Tween-80 fraction V mixture, however, have yielded information of great value in the study of tubercle bacilli. Two outstanding advantages are: (1) since the organisms are evenly distributed throughout the fluid, it is possible to grade and measure dosages and inocula with an accuracy impossible with the granular cultures; and (2) contrasted with the three- to four-week-old cultures formerly widely used in tuberculosis studies, in which many of the organisms have died, Dubos cultures develop quickly and contain mostly active young cells. With such cultures it is possible to infect mice by intravenous inoculation and to cause extensive and rapidly fatal disease in them whereas, using the older culture methods, mice had generally been thought highly resistant. Cultures injected intracerebrally, or fed once to the mice, also produced pulmonary disease. Pigmented mice were usually found much more susceptible than albino mice; a finding of interest in view of the greater prevalence and severity of the disease in pigmented human races.

It was found that neither age nor weight of mice affected their susceptibility to *initial* infection, but that, *once infection had occurred,* many factors, including age, weight, pigment, nutrition, etc., affected the further development of the disease. This finds a parallel in human experience.

It was also found that the bacilli from such cultures multiply enormously in the yolk sac of developing chick embryos and that a little such material, or sterile yolk mixed with the Tween-80 culture, would cause a rapid development of tuberculosis in mice, even if injected by the intraperitoneal (the least effective) route.*

Of special interest was the finding that, by combining intranasal instillation of doses of influenza virus *too small in themselves* to cause perceptible disease, with intraperitoneal injections of *subinfective* doses of the tubercle bacilli in Tween-80 medium, rapid and progressive pulmonary tuberculosis could be produced. This is of interest because extraneous disease (*e.g.*, influenza) as an unfavorable factor in human tuberculosis has long been known.

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* This is suggestive of the effect of the so-called spreading factor (hyaluronidase) of Duran-Reynolds, the effect of mucin on infections of mice with meningococci and typhoid bacilli, and the effect of a little broth on infections with streptococci (see Chapters 6 and 19).

It was found that virulent tubercle bacilli in the Tween-80 medium grew in distinctive longitudinal alignments, forming "ropes" or "cords" of bacilli, while avirulent variants, or those of low virulence such as B.C.G. (see page 753) tended to form amorphous aggregations.[26, 27, 28] The possible value of these observations in estimations of virulence is obvious, but they require further study.

Inoculation of Animals with Tuberculous Material. The possible value of mice in tuberculosis studies has been described above. In the traditional procedure the material from sputum or other material, prepared as described in the preceding paragraph, may be injected subcutaneously into the groin of guinea pigs. The development of tubercles in the animals is a slower but probably more sensitive means of demonstrating tubercle bacilli than microscopic search or cultural methods. If even a very few tubercle bacilli are present they will infect the lymph glands in the neighborhood of the injection and in three to six weeks the glands will be found enlarged and caseated (cheesy gangrene) and full of acid-fast bacilli. The spleen may also be invaded. If the lesion at the site of inoculation remains localized and does not progress, it suggests the action of an acid-fast saprophyte rather than that of a virulent tubercle bacillus. Animal inoculation has the advantage over the more rapid culture methods that it yields data on virulence, which the cultures do not.

Mycobacterium Leprae. In 1876 Hansen described acid-fast bacilli in lepromatous tissues. Morphologically they are indistinguishable from tubercle bacilli. Many workers have cultivated organisms resembling Hansen's bacillus from leprous lesions but none of these can produce leprosy. They are in all respects like the acid-fast saprophytes of the soil. Their relationship to leprosy is unknown. Burdon has shown that they have a different appearance from the bacilli in leprosy lesions when stained for fat.[29]

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Chapter 27

THE SPIRAL, FLEXIBLE BACTERIA (ORDER SPIROCHAETALES)

Although the organisms to be discussed in this chapter are included among the Schizomycetes, it will be seen presently that they differ markedly from most of the bacteria already discussed. In several respects they resemble some of the protozoa, especially trypanosomes (see page 894). The term spirochete is often used in a general sense to include all members of the order and will be so used here. It must be differentiated from the name of one genus of spirochete: *Spirochaeta.*

General Characters and Structure of the Spirochaetales. The order Spirochaetales is at present divided into two families: Spirochaetaceae, which includes three genera of relatively large, free-living, saprophytic spirochetes: *Spirochaeta, Saprospira* and *Cristispira;* and Treponemataceae, comprising three genera of relatively small organisms some of which are highly pathogenic: *Treponema, Borrelia* and *Leptospira.* Their nutrient requirements are complex. Only a few species have been cultivated on artificial media.

While the large saprophytic types differ greatly from the parasitic types in morphology, all of the spirochetes possess certain similarities and properties which entitle them to a position in this order and in the Schizomycetes. They are cylindrical in form. None forms pigments, spores, conidia, branches, filaments, cysts, sheaths, stalks or capsules. There are no demonstrable nuclei. They exist as single cells although some may form long spiral filaments consisting of several cells end-to-end. Dyar describes certain *Spirochaeta* as "multicellular spiral filaments."[1] Multiplication is by transverse fission. They are chemosynthetic. All are (*a*) distinctly *spiral* or *wavy* in form (Fig. 223), (*b*) *highly flexible* and (*c*) *contractile.* Motility in most spirochetes has been thought to be accomplished without flagella, their motion depending on their helicoidal form and their rotation around the long axis, as well as by slow snake-like bending movements. However, Mudd and his co-workers[2] demonstrated what seem to be flagella on certain treponemas by means of the electron microscope (Fig. 224). Flagella have not been seen on other spirochetes. Some (genus *Cristispira*) have a long, fin-like filament attached to one side; others have a septate or chambered structure; still others seem to have chitin-like, somewhat firm, axial filaments although this may be doubtful. From this brief description it is evident that spirochetes are sharply differentiated from the suborder Eubacteriineae and from the tribe Spirilleae of that suborder.

The spirochetes, while in general not much thicker than bacteria, are usually much longer, sometimes several hundred times as long; individuals as long as 400 or more microns having been described in saprophytic types. Spirochetes as a rule are not readily stained by ordinary dyes like methylene blue, although certain species are exceptions. When treated by Gram's method, those which respond are gram-negative. Special stains such as Giemsa's, which color protozoa, also

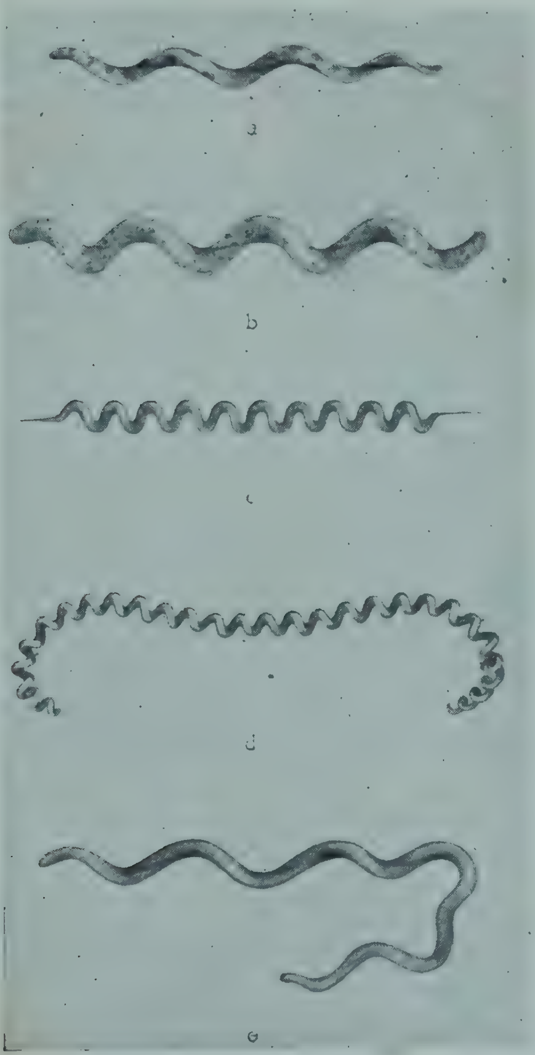

Fig. 223. Diagram of various types of spirochete. *a*, Spirochaeta; *b*, Cristispira; *c*, Treponema; *d*, Leptospira; *e*, Borrelia.

stain the spirochetes. The larger species are conveniently observed in unstained preparations, and the method of negative staining sometimes gives satisfactory preparations. The darkfield apparatus is most convenient for observation of the pathogenic species when fluid suspensions are available. For tissues, special silver-impregnation methods are used for staining. These will be described later. Granules, thought to be associated with reproduction, some of them Feulgen positive, have been seen (Fig. 224). Some species, especially saprophytic ones, contain many volutin and fat granules. Surrounding the cell is a definite membrane or periplast (doubtful in *Leptospira*). In *Saprospira* and *Critispira* the cell is divided into chambers by definite transverse, protoplasmic septa.

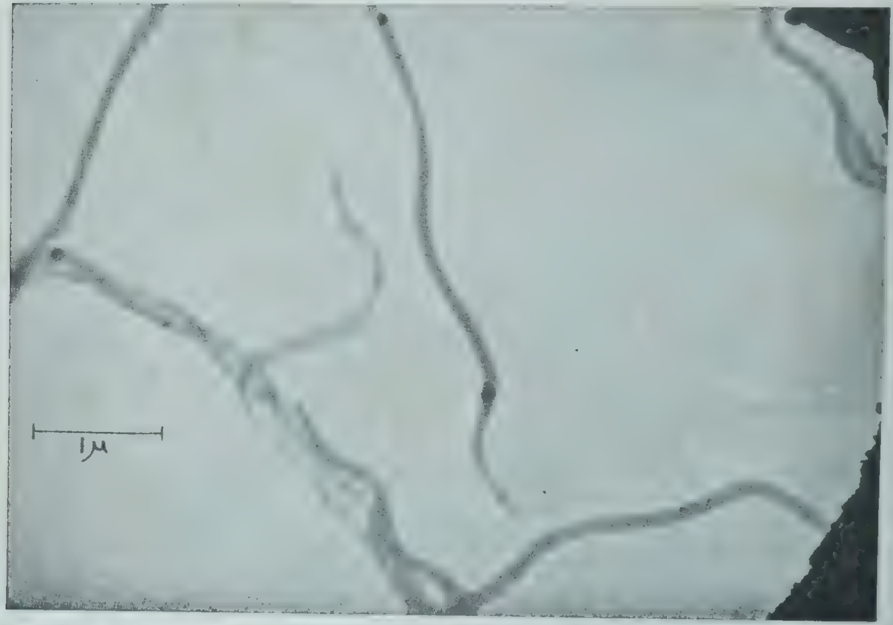

Fig. 224. *T. pallidum* (Nichols-Hough strain). × 14,000. Intertwined spirochetal cells. Granules, 40 to 90 $\mu\mu$ in diameter, are clearly shown within the protoplasm. One tuft of four structures resembling flagella is clearly seen. (Mudd, Polevitzky and Anderson, J. Bact., vol. 46.)

Filterability of Spirochetes. Certain of the spirochetes (*e.g.*, *Leptospira* and *Borrelia*) are readily filterable through porcelain or kieselguhr filters and this, of course, distinguishes them from true bacteria. It is thought by some authorities that the filterability of these organisms is due to the formation, through disintegration or otherwise, of minute granules. Possibly these are the supposed reproductive granules referred to earlier. Material which has been filtered and found, on microscopic examination, to contain no visible spirochetes, has later been shown to contain spirochetes by cultural methods.[3] However, microscopic examination of a drop or two of filtered fluid might easily fail to detect the presence of a few spirochetes in the greater, unexamined portion.

GENUS SPIROCHAETA

The name of this genus is derived from the name "Spirochaete," by which Ehrenberg, in 1833, designated a very large, spiral and flexible organism found free-living in stagnant water. This organism is now the type species of the genus *Spirochaeta* and is called *Spirochaeta plicatilis*. It is a slender spiral (0.5 microns in diameter) but often attains the relatively enormous length of 500 microns (0.5 mm.). What has been thought to be a distinct, wavy, axial filament around which the protoplasm seems to coil was shown by Dyar to be probably a staining artifact.[1] The ends are blunt (compare *Treponema,* etc.). Large granules of volutin and fat are present (Fig. 223). By means of phase microscopy Dyar has shown that the longer spirals are really made up of numerous short spiral cells end-to-end and separated by cell membrane which might give the impression of being septa in a single organism. Motility is by creeping and twisting movements. The organisms may be found in sewage and stagnant water. Pure cultures have been made on medium containing extract of leaves, and on 1.5 per cent agar with red blood cells. They are aerobic and grow in a *p*H range of 6 to 9 at around 26° C. They grow as a thin film on the agar, rarely forming ordinary colonies.[1] They are entirely harmless to man as far as is known (Fig. 225).

GENUS SAPROSPIRA

This genus resembles the foregoing in that the organisms are found free-living in water, have blunt ends, and are curved and wavy. The organisms are said to be thicker than *Spirochaeta plicatilis,* having a diameter of about 1 micron, but are shorter, seldom exceeding 85 microns in length. Saprospiras possess no demonstrable axial filament but appear to have septate or chambered protoplasm. This could represent separate cells as in *Spirochaeta. Saprospira* also possess a distinct cell membrane and contain volutin granules. Some species are found in the intestinal tract of mollusks. The group is very little known. Saprospiras have never been cultivated. Dyar doubts that *Saprospira* and *Spirochaeta* represent separate genera. Only two or three species are known.

GENUS CRISTISPIRA

In most respects the cristispiras resemble the saprospiras. They have a wavy arrangement, blunt ends and a septate protoplasm without axial filament (Figs. 223 and 226). The cell membrane is distinct.

Motility is active. Their length ranges from 26 to 120 microns and their diameter from 0.5 to 3 microns. The distinct cell membrane stains like chitin; a protozoa-like character.

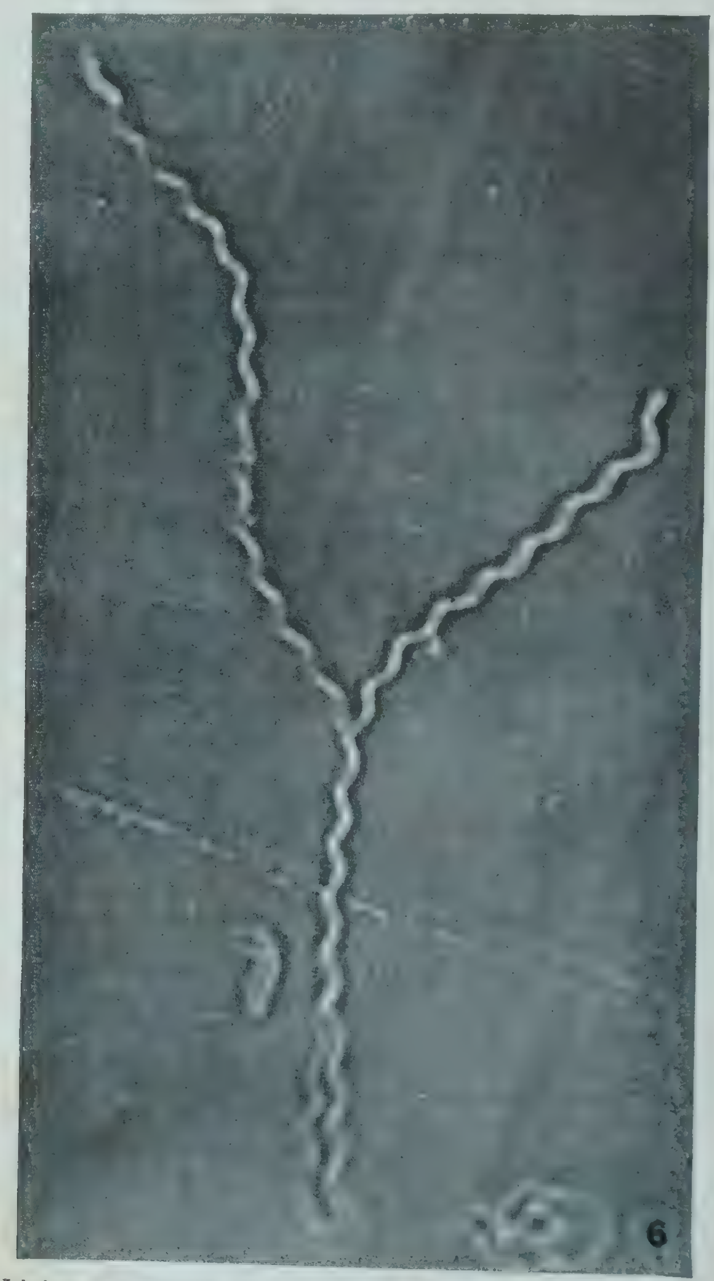

Fig. 225. Living spirochetes photographed with the bright-contrast phase microscope. × 1,800. (American Optical Company.)

There are two matters of especial interest connected with cristispiras. One is their habitat, which is restricted to the intestinal tract of oysters and other lamellibranchs or to a hyaline structure, called the *crystalline style,* in the esophagus of these mollusks. The other is the presence of a sort of keel or membrane, which winds spirally about the organism one edge free, the other attached to the cell. This structure is suggestive of the undulating membrane seen in one genus of pathogenic protozoa, the trypanosomes (see page 894). The generic name is derived from this membrane or *crista.* About twenty species have been described. The organisms are entirely harmless. None of the cristispiras has ever been cultivated.

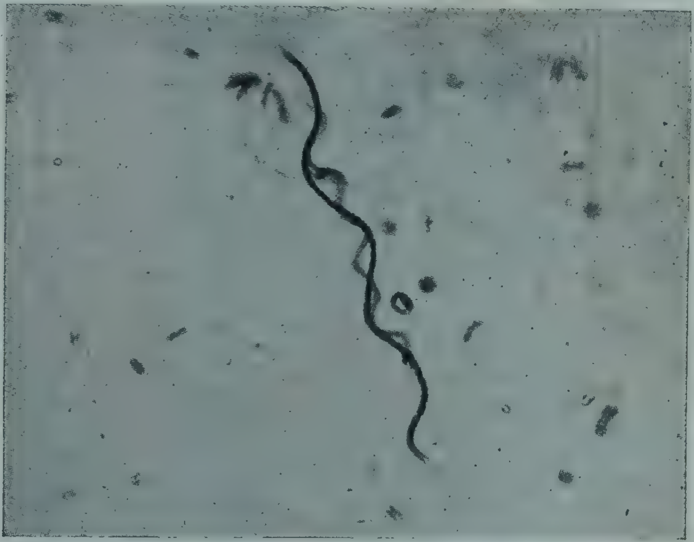

Fig. 226. *Cristispira balbianii,* showing crista. (Hideyo Noguchi.)

GENUS TREPONEMA

Treponemas are much smaller than any of the spirochetes so far discussed. They are often exceedingly slender (0.25 microns to 0.4 microns in diameter), seldom exceeding a length of about 15 microns. Their size is therefore comparable with that of true bacteria. Their cell structure is much less perfectly demonstrable than is that of the four preceding genera. The organisms have neither crista nor septa. The eight to fourteen spirals found in treponemas are close and regular unless the protoplasmic contractions change them. The ends of the organisms are drawn out into extremely fine fibrils which may represent either prolongations of the axial filament or merely remnants of the fine thread of cytoplasm connecting the organisms before fission is complete. These terminal fibrils have no function in the motility of the organism. Motion may depend on flagella but the propeller-like action of the spirals when the treponemas rotate doubtless plays a major role.

Treponemas are not easily stained. Indeed, the first treponema to be described, that causing syphilis (Schaudinn, 1905), was named *Treponema pallidum* because of its pale appearance. Other methods are, therefore, used to demonstrate them microscopically. One is the method of negative staining; another, widely used to examine exudate from lesions of syphilis for diagnostic purposes, the darkfield apparatus (see Chapter 3); and a third is termed "silver impregnation." For this, several methods are available, a useful one that of Fontana.* In this method ammoniacal solutions of silver salts are allowed to penetrate the cells and metallic silver is then precipitated there by means of a reducing solution. The organisms appear black against a yellowish background (Fig. 227).

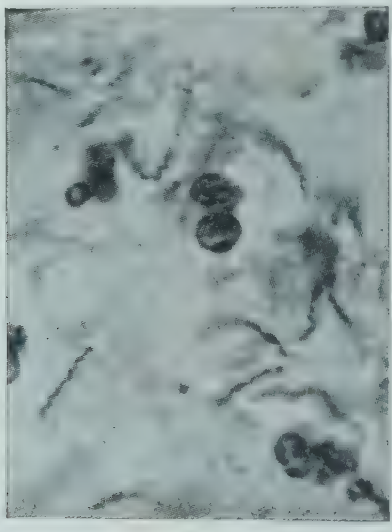

Fig. 227. Treponemas of yaws in tissues stained by silver impregnation. (Ferris and Turner.)

T. pallidum is a relatively fragile organism. It has never been cultivated in artificial media. Some similar species have been cultivated. but none of these is able to cause infection. *T. pallidum* survives, under

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* *Fontana's silver stain for spirochetes:*

Solution A	
Tannic acid	5 gm.
Distilled water	100 ml.
Solution B	
Silver nitrate	5 gm.
Distilled water	100 ml.

To 90 ml. of solution B slowly add concentrated ammonium hydroxide till a brown precipitate forms and *just* re-dissolves. Then add, very cautiously, more of solution B till the fluid shows a faint, permanent cloudiness. This "keeps" well.

Flood smear with solution A and steam for about 30 seconds. Wash with water and flood with solution B, steaming for about 30 seconds. Wash and blot.

ordinary circumstances, for only very short periods outside of the tissues of man or experimentally infected animals. Hence nonvenereal infection of man is rare. However, Turner and his associates have shown that, when quickly frozen and maintained at —76° C. by means of solid carbon dioxide, syphilis spirochetes, as well as other organisms, will remain viable and fully infectious for years.[4] The organisms do not long survive ordinary drying.[5] In citrated blood stored in "blood banks" they quickly die out.[6]

T. pallidum is pathogenic for several animals, notably rabbits, and these animals are serving a useful purpose in such studies as the relationship of treponemas of yaws, syphilis and a natural disease of rabbits, as well as the therapeutic effect of penicillin in syphilis.[7, 8, 9, 10]

Syphilis. Syphilis, caused by *Treponema pallidum,* is primarily a venereal disease, *i.e.,* is transmitted chiefly by sexual intercourse. When so transmitted and when it develops typically it begins, within a month after exposure, as a small ulceration on the mucosal surface of the genitalia. The ulcer increases in size, becoming rather hard and flat. Upon removal of the crust of serous fluid oozes from the surface and, upon examination with a darkfield apparatus, may be found swarming with *Treponema pallidum.* When syphilis is acquired through kissing, the chancre may occur on the lip. This ulcer, oral or genital, is spoken of as a "primary lesion" or *hard chancre* (pronounced shank'er). It tends to heal spontaneously due to the development of antibodies[8, 11] after two or three weeks and the victim may believe himself cured. Many proprietors of quack remedies and vicious charlatans preying upon the fears and ignorance of their fellows have thrived upon this fallacy.

What really happens is that by this time a certain degree of immunity develops. The treponemas have *long since migrated* from the primary lesion and been carried all over the body, localizing in various organs, particularly the liver, spleen, walls of arteries, heart, brain, skin and mucosal surfaces, setting up "secondary lesions" after two to four months. When situated on the skin these appear as red blotches or an extensive rash and may be very infectious. They may occur on any part of the body and teem with treponemas. Infectious white patches may also appear in the mouth and genitalia. In such conditions of the mouth kissing of other persons results in infection of the lips, tongue or gums of the latter. The teeth may loosen and come out, as well as the hair. The latter sometimes becomes patchy looking and is often spoken as as "moth-eaten hair." After a time (weeks or months) these outwardly visible, *secondary* lesions slowly disappear in great part and the patient may again believe himself cured, Sometimes spontaneous cure actually may occur but this is probably rare.

In untreated syphilis the treponemas usually slowly cause extensive *tertiary lesions* called *gummata* in various internal organs and also on the skin. These tend to heal and form scars as the process continues. The liver becomes damaged and scarred (syphilitic *cirrhosis* of the liver) and bulges appear in the aorta where lesions in the layers of the vessel have weakened it. These bulges are called *aneurysms*. When they burst, death from hemorrhage often ensues. Gummata also occur in any of the bones.

The treponemas also damage the brain and spinal cord. Various nerve centers are slowly destroyed and characteristic forms of insanity and paralysis result. Death follows, sometimes after a period of many years.

This picture of syphilis is not pleasant and, indeed, the disease is one of the most insidious and dangerous. Many thousands of persons die annually of this disease in the United States. Many cases exist which do not come to the attention of the recorder.

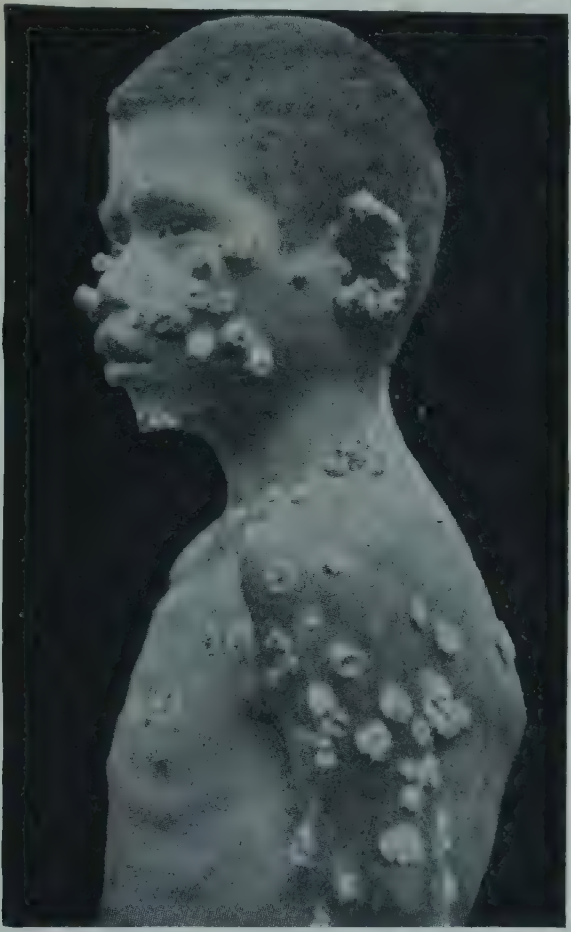
As in the case of gonorrhea, prostitution and sexual promiscuity are the chief means by which syphilis is spread. In spite of renewed efforts by federal, state and local authorities to educate the public to the dangers of syphilis and to enlist the aid of legislatures, medical and civic authorities in preventing its spread, many new cases appear each year. However, syphilis is no longer a secret and sneaking enemy of the shadows, but it has been brought into the light by public opinion, by open, matter-of-fact discussion in newspapers and public meetings. The medical officers of the armed forces have done much in the campaign of education and prevention, and greater enlightenment on this subject is one of the many benefits to the country as a whole from military service. The United States Public Health Service and all local health agencies are active in fighting all venereal disease.

Aside from prophylactic measures which need not be discussed here, it may be controlled to some extent by measures directed against prostitution and by early treatment, but the treatment is long, expensive, painful and involves arsenic, a dangerous poison. The use of penicillin has given dramatic results,[12] but is still in an experimental stage.

The diagnosis of syphilis, after the disappearance of the primary lesion in which the spirochetes are demonstrable microscopically by means of the darkfield apparatus, is made by means of the Wassermann test or a precipitin test such as the Kahn, Eagle or Kline test (see page 325).

TREPONEMA PERTENUE

Yaws. Yaws is an ulcerative disease, common in, and restricted to, tropical countries. The earliest lesions usually appear on the skin of the feet, legs, and arms (Fig. 227*a*). It is commonest in children up to 15 years of age. It is not a venereal disease, evidence obtained by Turner and Kumm indicating that it may be insect-borne.[13] Close contact with ulcers appears to be a common means of transmission.[14] Yaws occurs only in man. The causative organism, *Treponema pertenue,* is morphologically indistinguishable from *T. pallidum.* Patients with



ig. 227*a*. Yaws lesions. (Napier: Principles and Practice of Tropical Medicine, The Macmillan Co.)

aws give positive Wassermann and precipitin tests and respond to the ame treatment (salvarsan, arsphenamine, etc.). Yaws is not so danerous a disease as syphilis but causes very extensive syphilis-like esions nevertheless. There is little involvement beyond skin and bones. he spirochetes are found in the early lesions. Natives of the tropics re said to recover from it without treatment.

Cross Immunity in Spirochetal Disease. Yaws illustrates a very ineresting relationship of one spirochetal disease to another. An attack of yaws often apparently confers some degree of immunity to syphilis. The reverse may occur but is not demonstrated. Does yaws represent merely a different clinical manifestation of syphilis; is it a different disease; or is *Treponema pertenue* a modified type or variant of *T. pallidum,* changed by contact with a different race of people so that it has lost virulence, much as smallpox virus loses virulence for man on contact with cattle? At present, there is no definite answer to these questions, but Ferris and Turner[15] have shown that the spirochetes of yaws produce, in rabbits, lesions which are readily distinguishable from those produced in rabbits by *T. pallidum,* indicating that there are definite differences between the organisms. Furthermore Turner, McLeod and Updyke[9] showed that rabbits having received intratesticular inoculations of *T. pertenue, T. cuniculi* or *T. pallidum* were later found to have considerable resistance to each of the heterologous species. As stated by these authors: "The ability of an organism (*T. cuniculi*), causing a natural disease of rabbits, to produce an increased resistance to syphilis is of particular interest." These observations are illustrative of the *general proposition* that closely related species of microorganisms often contain antigens in common which may be of the greatest value in studying taxonomic relationships as well as in developing new methods for immunization against disease.

GENUS BORRELIA

While there are differences in morphology, pathological action and resistance to external conditions, the borrelias resemble the treponemas closely in many respects, such as cell structure and staining peculiarities. However, many borrelias can be stained readily by Gram's method or by means of a special polychrome stain made by mixing eosin and methylene blue (Jenner's stain, Wright's stain*). They often have a less definite spiral form, being more wavy and open (Fig. 228) especially in stained preparations. In death they seem to relax and lose their regular, coiled form. They are also somewhat thicker and coarser-looking than the treponemas. Cultivation has been accomplished although it is not very satisfactory.

Commensal species of *Borrelia* occur, often in large numbers, in the mouth (*Borrelia macrodentium, B. microdentium*) and on the external genitalia (*B. refringens*). Some of these so closely resemble *Treponema pallidum* in appearance as to create confusion at times in the diagnosis of syphilis by microscopic methods. They appear to be harmless. They are sometimes classed as *Treponema.* Leeuwenhoek probably was the first to observe these. The majority of the pathogenic species, like *B. recurrentis,* are blood parasites causing fever of a relapsing nature.

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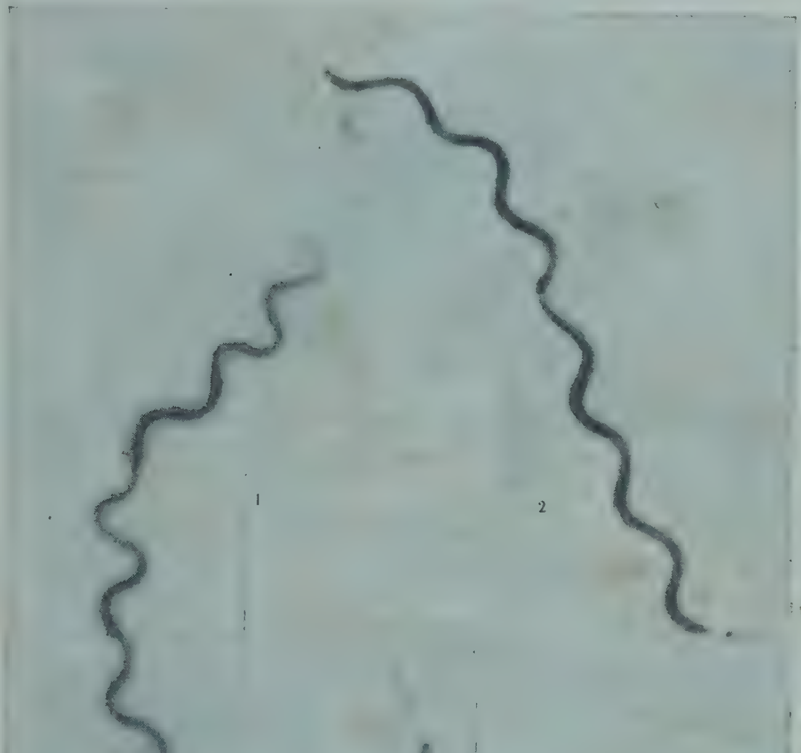
* These stains are commonly used for staining blood films to demonstrate the protozoa of malaria. They are available commercially.



ig. 228. *Borrelia novyi,* prepared by electrodialysis and dilution after having stood 48 hours in distilled water. (From Lofgren and Soule, J. Bact., vol. 50.)

The blood-inhabiting borrelias differ from the treponemas in being ransmissible from one person to another by certain insects (*Ornitho-*

dorus ticks or lice), in which they survive for considerable periods although often in an apparently unrecognizable form so minute that they will pass porcelain filters. This lends support to the view that they pass into an invisible granular stage which is filterable. Filtered blood of relapsing-fever patients also transmits the disease.

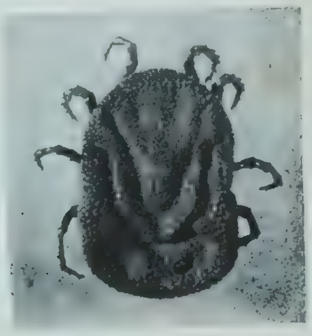


Fig. 229. African relapsing fever tick, *Ornithodorus moubata.* × 3. (From Herms, "Medical and Veterinary Entomology," by permission of The Macmillan Co., publishers.)

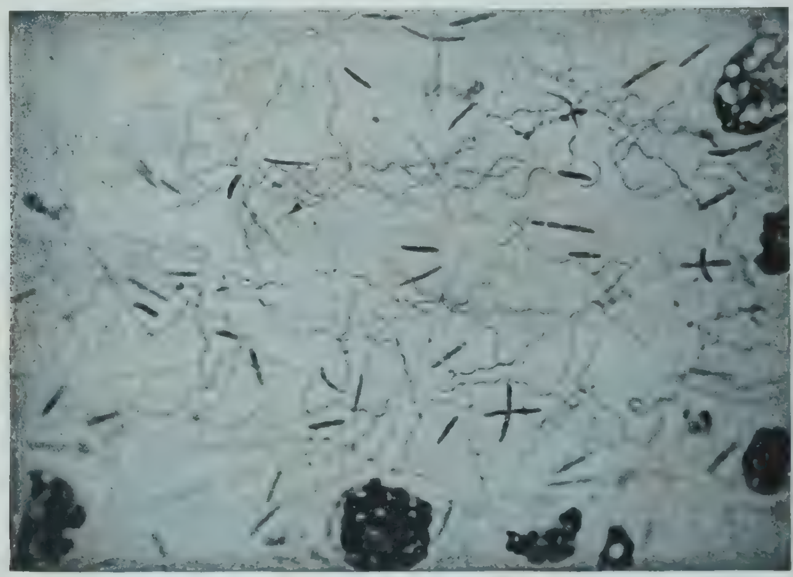


Fig. 230. Vincent's angina. Stained smear showing fusiform bacilli and spirochaetal forms. (×900.) (Todd and Sanford.)

Relapsing Fever. Relapsing fever is characterized, as the name indicates, by a series of relapses after the first fever subsides, which it often does very suddenly. The disease in America is transmitted by lice and ticks, in Central Europe, it is transmitted by lice; and in tropical and subtropical countries it is mainly tick-borne (Fig. 229).

The organisms are found in the blood of patients during the febrile periods, but during the afebrile interludes seem to retreat to internal foci in the liver, spleen and lymphatics.

In the louse the spirochetes undergo a developmental cycle of five or six days during which they are neither infective nor visible. After this period they can be recognized as such. They are not transmitted by the bite or feces of the louse, but by crushing the insect on the skin. In the tick a similar cycle occurs but the tick can infect by biting and by their feces, as well as by being crushed. True bacteria do not undergo such cyclical changes.[16]

Trench Mouth. Some anaerobic species of *Borrelia* are found in the mouth (notably *B. vincentii*) associated with ulcerative conditions ("trench mouth," or *Vincent's angina*).[17, 18] They may be seen readily in gram-stained smears from such conditions, mixed with fusiform bacilli (Fig. 230). The name "trench mouth" originated in the frequent occurrence of outbreaks of the disease in soldiers in trenches during World War I. It is presumably transmitted by unclean eating utensils and other articles which carry saliva directly from mouth to mouth. It may be associated with dietary deficiencies.

GENUS LEPTOSPIRA

Leptospira are the smallest of the spirochetes. Their spirals are so fine and so closely wound that, when observed in the darkfield, only the outer curves of the spirals are seen and the organisms appear like strings of minute, illuminated beads. The leptospiras are further characterized by being bent into a hook at one or both ends (Fig. 231). Their motion consists of a writhing and flexing movement and a rapid rotation around the long axis. Their progression from place to place is rapid and can be readily explained on the basis of their screw-

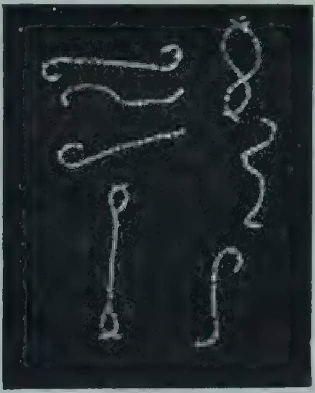


Fig. 231. *Leptospira icterohaemorrhagiae.* Appearance of organisms in the darkfield. (Zinsser, Bayne-Jones, "Textbook of Bacteriology," D. Appleton-Century Co., publishers.)

like form and their rotation. They may be cultivated readily in various media, a representative one being that devised by Chang.[19] Most such media are mixtures of nutrient agar and serum.

Leptospiras are chiefly saprophytic, aquatic organisms, which are found in river and lake waters, in sewage, and in the sea. Occasionally, they are found in the normal mouth. As shown by Bauer and Sawyer[3] and others, they may be cultivated in pure culture from some pond or swamp waters by the simple procedure of passing the water through a porcelain or kieselguhr filter (through which they readily pass), and then adding (aseptically) 10 per cent sterile serum, 0.8 per cent salt and melted agar to yield 0.2 per cent. Leptospiras may frequently be demonstrated in river, lake, or well water by adding to a liter of it the yolks of three eggs or a few grams of fecal suspension and incubating in a broad, flat dish at room temperature. These cultures are mixed.

Relation Between Pathogenic and Saprophytic Leptospiras. Leptospiras (*Leptospira icterohaemorrhagiae*) morphologically and culturally indistinguishable from the harmless species found in nature occur in the blood and urine of patients suffering from Weil's disease (infectious *hemorrhagic jaundice*). Several other clinically similar conditions are due to other species. For the present we may only speculate on the relationships between the saprophytic and the pathogenic forms. Authorities disagree as to whether the pathogenic species are derived from the saprophytes through a process of adaptation to life in a host by passage from one host to another with resulting increases in virulence; or whether the two are entirely separate kinds of leptospiras. There is experimental evidence on each side.

For one thing, the pathogenic types can survive free in wet soil and in water for long periods and, since *they can also infect* rats, may be spread about by the urine of these ubiquitous animals and thus appear to be "free-living" water forms when their source is not known. Thus, for example, rat-injection experiments with stagnant or dirty water containing supposed "water leptospiras," in which infection is obtained, could easily be vitiated by unsuspected contamination of the water with pathogenic strains from rats.

Weil's Disease. This disease is quite widespread. It is properly called spirochetal jaundice or infectious hemorrhagic jaundice and clinically resembles yellow fever in many ways. It is common among persons who spend much time in wet, poorly drained situations such as badly constructed mines, wet trenches during war, and the bilge of river and lake boats, especially if rats abound there. In Hawaii the incidence of the disease in sugar-cane workers seems directly related to average annual rainfall.[20] It has been found with increasing frequency in the United States and is doubtless world-wide in distribution. The spirochete may be readily demonstrated in wild rats by darkfield examination of mashed, fresh kidney or liver. Ward and Turner,[21] in a survey of Baltimore poultry-dressing, meat and related establishments, found much evidence of it among employees (17.3 per cent). Rats infested the premises. They also found active infection in persons using water from a well near an airplane factory in that city and demonstrated the leptospiras in the water. Rats were abundant around the well. As shown by Meyer and others,[22–28] dogs may likewise transmit this organism, as well as a similar species, *L. canicola* which produces fatal jaundice in them, to man.

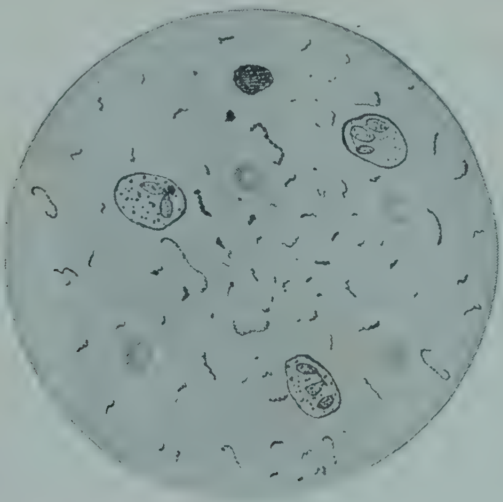


Fig. 232. Leptospiras undergoing lysis in Pfeiffer's phenomenon (× 900).

Leptospira icterohaemorrhagiae occurs in the blood and urine of human beings ill with the disease and may be cultivated from them by methods given above. It is readily transmitted from man to man by polluted water and is thought to get into the body also by way of the feet when one remains long in infected water.[29]

Immunity in Weil's Disease; Pfeiffer's Reaction. Shortly after the onset of infection with *Leptospira icterohaemorrhagiae,* lytic antibodies begin to appear in the blood stream. These increase in concentration until the serum of the person is capable of protecting him against large doses of the leptospiras. The agglutinin titer increases markedly and is useful in diagnosis.[30] Cultures in fluid media, concentrated by centrifugation, killed with formalin and stained, are used as antigen. *L. canicola* and *L. icterohaemorrhagiae* are serologically distinct.[31]

The phenomenon of cytolysis may be beautifully illustrated with such serum. A drop of culture of *Leptospira icterohaemorrhagiae* is mixed with some of the patient's serum which has been heated at 56° C. for thirty minutes to destroy the complement in it. The leptospiras remain unchanged in any visible way. One can then demonstrate that they have been *sensitized* by the *lytic* antibodies in the serum. When fresh, complement-containing serum of some normal animal (*e.g.*, guinea pig) is added, the leptospiras may be seen, by means of a "darkfield" apparatus, to swell, granulate, and undergo various distortions and finally to disappear (Fig. 232).

Active, artificial immunization is said to be effective in inducing potent antibody production against *Leptospira icterohaemorrhagiae.* The whole matter of pathogenic spirochetes from all viewpoints is well reviewed by Stavitsky.[32]

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Section III

THE TRUE BACTERIA (ORDER EUBACTERIALES)

(Suborder Eubacteriineae)

INTRODUCTION

In the preceding section we have presented a description of microorganisms which are frequently encountered by the microbiologist and which are studied in much the same manner as true bacteria and in many ways resemble them. However, the yeasts and molds are readily seen to be unlike "fission-fungi" in having demonstrable nuclei, sexual phenomena and in being of a much greater order of magnitude. They are true fungi (Eumycetes). The so-called higher bacteria discussed in Section II have no nuclei, no obvious sexual phenomena and the *diameter* of their cells or filaments is generally like that of true bacteria, *i.e.,* of the order of 0.1 to 2 microns. The filamentous higher bacteria never attain lengths comparable with the filamentous molds. They are much more like bacteria than like molds. But they are distinguished from the typical true bacteria (Eubacteriineae), discussed in this section, by various special structural or physiological characters such as branching and conidia formation in the Actinomycetales; sheaths, stalks and iron deposition and sulfur granules in the Chlamydobacteriales and Caulobacteriineae; filaments, photosynthesis, and sulfur granules in the Rhodobacteriineae, and so on.

In the suborder of Eubacteriineae, which is introduced in this section, these complexities of structure and function are absent. The single, separate, individual cell is the entire plant, independent of its fellows. The structure is a simple sphere, a rod or a short rigid curve or spiral. Cell fission does not, as is the case in some of the higher bacteria, merely enlarge the whole plant; it creates two, new, independent, complete plants.

It is the purpose of this section to describe simple bacteria of this general type which are found free-living in the soil, water, food, dairy products, etc., or in the intestinal tract, skin, etc., of man or animal; and to briefly discuss the relation of these organisms to each other, to industry, to agriculture and to disease. Most of the bacteria which regularly cause disease will be discussed in a subsequent section (IV).

Chapter 28

THE AEROBIC SPOREFORMING RODS (GENUS BACILLUS*)

All of the organisms in this genus are straight rods capable of forming heat-resistant spores and having dimensions ranging around 1.5 microns in diameter and 10 microns in length (Fig. 233). Most of them are motile. Nearly all species are gram-positive but some are more strongly so than others. They are typically strictly aerobic. There is only one dangerous pathogen for man and animals in the genus, *Bacillus anthracis*. The remainder are mainly harmless saprophytes. However, occasionally some of the latter species have been isolated from pathologic conditions in which they appeared to be the causative agent; this is only one of many illustrations of the fact that bacteria which are habitually saprophytic may at times become adapted to a parasitic mode of life.

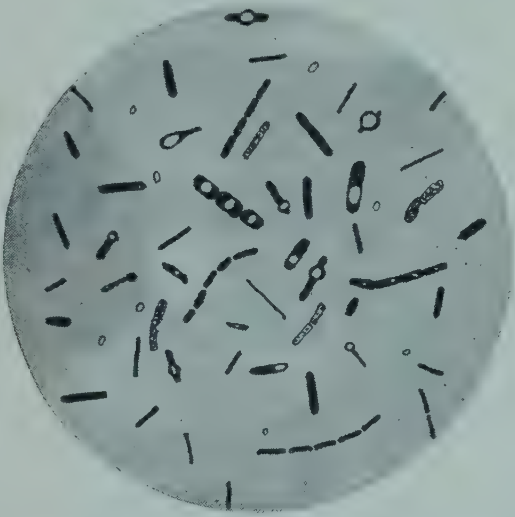

Fig. 233. Various types of sporeforming rods of the genus *Bacillus* (× about 1000).

The genus, as at present classified, comprises 33 species, subdivided into groups on the basis of optimal growth, temperature, biochemical reactions, size, shape and location of spores, motility, etc. Because all of the species are similar in fundamental respects, it seems advisable to do no more than outline the major peculiarities of the group as a whole and point out a few features that appear to be of interest from a practical and philosophical point of view. Because of their large size and widespread habitat, several of the best known species were observed very early and are therefore of historic interest.

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* In order to avoid confusion of names, the student is urged to re-read the paragraph on page 68 concerning the use of the terms "bacillus" and "bacterium."

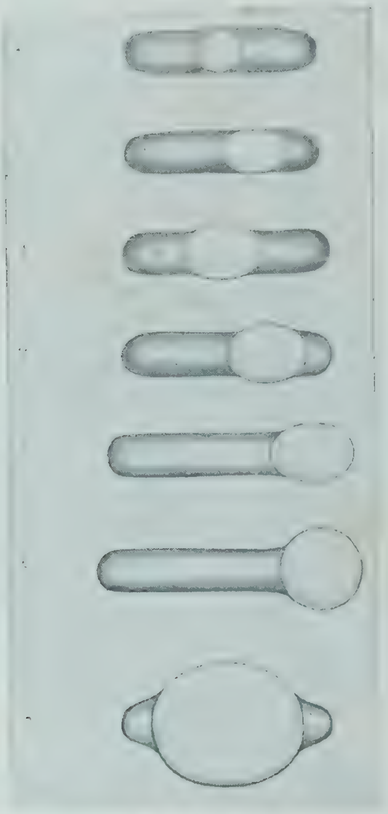

Fig. 234. Types of sporulation. *1*, Spherical; central; cell not swollen. *2*, Oval; excentric; cell not swollen. *3*, Oval; central; cell swollen. *4*, Oval; excentric; cell swollen. *5*, Oval; terminal; cell swollen. *6*, Spherical; terminal; cell swollen. *7*, Large, oval, central.

Distribution and Functions of Bacillus. The spores of these bacteria are ubiquitous in soil, dust, and water. Observations of dust storms which occurred about 1935 in the northwestern part of the United States made it clear that spores of these bacteria and other forms of life are carried thousands of miles by currents of air, while Darwin, as early as 1831, on board the "Beagle," noted that the dust blown many hundreds of miles out to sea from Africa contained spores, some of them doubtless bacterial (*infusoria;* Ehrenberg).

It is not to be wondered at, therefore, that sporeforming, aerobic bacilli are often a source of embarrassment to the unwary bacteriologist who finds them multiplying unwanted in his cultures. They, with molds and micrococci, are among the most frequent weeds of the microscopic garden. It requires assiduous care and constant vigilance to exclude them. It was the heat-resistant spores of such organisms that misled Needham, Pouchet and others to support the view that life began spontaneously in the infusions which they *thought* they had sterilized by heating. Even experienced bacteriologists are sometimes embarrassed by their appearance in supposedly sterile material or pure cultures of bacteria. This is usually due to carelessness in the sterilizing room, "short-cuts" in heating processes, etc.

If we view evolution as a means of disseminating, preserving and multiplying a given type of living creature, the sporeformers would seem to be a favored group. They can utilize a wide variety of substances as food. A common property is that of hydrolyzing proteins like gelatin, coagulated blood, and dead plant and animal tissues. They also hydrolyze many different carbohydrates, glucosides, alcohols and organic acids. At least one species can utilize carbolic acid (*Bacillus closteroides*). Bacilli are thus seen to be of great importance as scavengers.

Spores and Sporulation. A peculiarity of the sporeforming, aerobic bacilli is their inability to form spores under *anaerobic* conditions. This is of importance in the study of anaerobic sporeformers, for the two types may be separated by anaerobic incubation followed by heating. The aerobes will not survive. Knaysi has made a careful study of the relation of environment to spore formation by *B. mycoides,* a common dust bacterium fairly representative of the mesophilic group. The effect of accumulated growth products is inhibitory rather than favorable to sporulation. The presence of oxygen, in artificial cultures at least, favors sporulation by retarding or preventing accumulation of acid and other partially oxidized growth products. The optimum *p*H for sporulation is 6.6 to 6.8. Vitamin B favors sporulation in synthetic media indirectly because it promotes decarboxylation of acid by-products. Drying has no favorable effect on sporulation and may inhibit it. Viable cells seem to be stimulated to sporulation by suspension in distilled water.[1] Apparently the spore contains no reserve source of energy as do seeds or plants.[2]

Fat globules are readily seen in cells of aerobic sporeformers cultivated on carbohydrate media and stained by Burdon's method.[3] According to Knaysi these appear to serve neither the spore nor the vegetative cell as reserve energy.[2] Their exact function is not clear.

Studies on the nature of heat resistance in the genus *Bacillus* have shown that, in general, the species having the most heat-resistant spores have the highest optimum or maximum growth temperatures, *i.e.,* are thermophils; species with low optimum growth temperatures have the least thermo-resistant spores; while non-thermophilic species with maximal growth temperatures between 50 and 60° C. have spores with intermediate heat resistance. The division between these groups is not sharp. Lamanna points out that organisms, whether spore-formers or not, which grow well at low temperature (0–20° C.) have little or no tolerance of temperatures of 45° or even as low as 30° C., while organisms which can grow at about 45–50° C. show considerable resistance to higher temperatures and many will survive pasteurization in milk (62° C. for 30 minutes).[4]

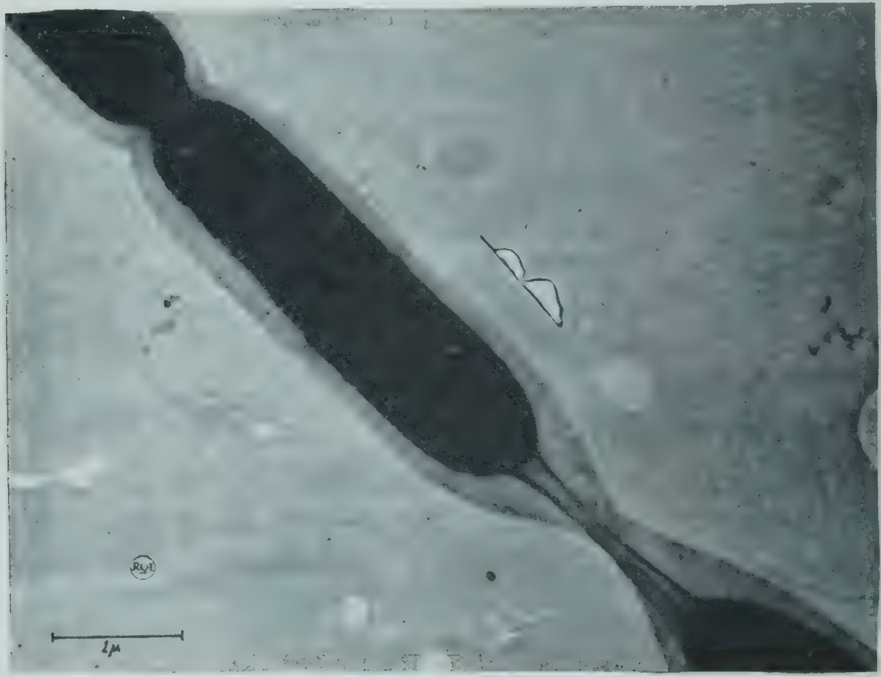

Fig. 235. Electronograph of Bacillus sp. Note cell membrane, flagella, dense cytoplasm and connecting bar of cytoplasm or cytoplasmic membrane between dividing cells. Note also the plate-like thickening near the center of the large cell where division is beginning. (Courtesy of Radio Corporation of America.)

Types of Sporulation. Much importance has been attached to the location and size of spores as a differential character among species of *Bacillus*. However, biochemical and other characters are of equal if not greater taxonomic value.[5] Spores are said to be *terminal* when they are absolutely at the end of the rod. When not exactly terminal or definitely central, they are designated as *subterminal* or *excentric* (Fig. 234). It is sometimes very difficult to determine the exact position of the spore in the cell. In some species the spore is small, having a diameter less than that of the rod. In others the diameter of the spore is much greater than that of the bacillus. Spores may be spherical or oval in shape. The cell, containing a spore, is often called a *sporangium.*

Structure of Bacillus. Considerable advances in our knowledge of the structure of certain representative members of the genus *Bacillus* have been made by means of the electron microscope. A picture of *B. cereus* by Johnson shows very clearly not only flagella, form and size, but the distinct cell wall, the protoplast of the cell proper, the cytoplasmic membrane and a plasmodesmid and cell wall stretched almost to the breaking point between two dividing cells. A dense band and a notch near the center of one cell appear to "represent very early changes presaging cell division."[6]

By comparing certain individual cells of *Bacillus megatherium* as pictured by light and by electrons, Dubin and Sharp concluded that the light microscope fails to reveal an outer structure of the cell wall which is evident in electronographs. The inner portions of the cell are dense, while the outer portion, revealed by the electron microscope, is less dense.[7] Interesting details of morphology have also been revealed by Mudd, et al., by means of the electron microscope[8] (see Fig. 235).

BACILLUS ANTHRACIS

Only one species, *Bacillus anthracis,* is very pathogenic to man. It rather closely resembles *B. subtilis,* a common saprophyte, described later in this chapter, but may be distinguished by the characteristics listed below:

Differentiation of B. anthracis and B. subtilis

B. anthracis		B. subtilis
Motility	None	Active
Growth in broth	Clear broth, cottony tufts of growth at bottom	Pellicle; turbid broth
Gelatin liquefaction	Very slow	Rapid; complete

Anthrax. This is primarily a disease of farm animals but it is transmissible to man. It has been known since antiquity and is probably referred to in the Bible (Exodus IX:9).* In man, the organisms most commonly gain entrance from soil or dust to the body through a cut in the skin. They first *localize* at the point of entrance, forming a very rapidly progressive, angry, inflamed pustule (*malignant pustule*), which, when well developed, is covered with a black crust. This pustule teems with anthrax bacilli. It not infrequently heals, but in other cases the bacilli invade the blood stream, multiply enormously, and are spread through all the organs of the body where they tend to form local lesions which serve as further centers for dissemination unless the leukocytes and other defensive mechanisms of the body overcome them. Unless the infection is vigorously treated the organism has been said to produce death by such great multiplication in the blood as to block the blood vessels. Probably other factors are equally important in causing fatal effects.

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* It is interesting to note this as an early, if not the earliest record of bacterial warfare: "and it shall become small dust in all the land of Egypt and shall be a boil breaking forth with blains upon man and upon beast throughout all the land of Egypt."

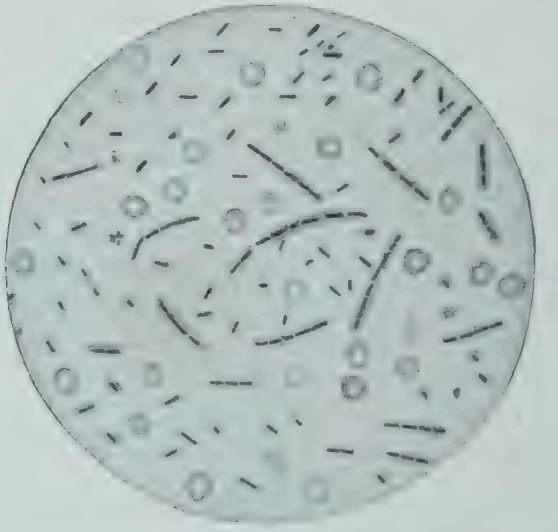

Fig. 236*A*. *Bacillus anthracis*. Smear from fresh blood showing capsules. The circular objects are erythrocytes. No spores are seen (× 900).

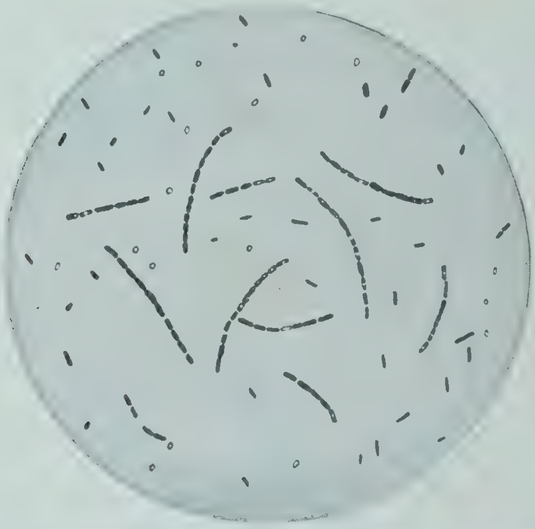

Fig. 236*B*. *Bacillus anthracis*. Smear from aerobic culture showing spores (× 900).

An inflammatory factor producing damage to the tissues appears to be formed by the organisms. This permits dispersal of spores from a site of inoculation, suggesting the action of a spreading factor such as hyaluronidase. The inflammatory factor also interferes with blood clotting and inhibits the action of leucocytes. It is of especial interest that the capsule produced by the organism *in an infected animal* (not in vitro) appears to be associated with the inflammatory factor[9, 10] (Fig. 236*A*).

When animals die of anthrax, there exudes from the body openings, for some time after death, a bloody fluid which teems with anthrax bacilli. Although *Bacillus anthracis,* like other aerobic sporeformers, does not form spores under conditions of low oxygen tension such as occur in the blood vessels, the bacilli in these bloody discharges quickly sporulate on exposure to air (Fig. 236*B*). These may remain alive on the ground in pastures for many years, constantly infecting cattle which graze there. Certain pastures in France have thus come to be known as "anthrax pastures." Postmortem examinations of animals suspected of having died of anthrax, should, therefore, never be made in the field. There is too great a danger of infecting many other animals and man since all of the blood and organs of the carcass contain billions of the bacilli which will sporulate as soon as the body is opened. It is sufficient to excise an ear, or make a culture from some of the bloody exudate. The animal should be buried or, better still, burned *on the spot.*

Animals may be artificially immunized by the injection of attenuated organisms, following methods based upon those devised by Pasteur (see section on immunity, page 334).

BACILLUS SUBTILIS

B. subtilis is the type species of the genus and is one of the commonest of aerobic sporeformers. It is found in dusty places everywhere and especially in hay. If hay be soaked in warm water for a day or two, the water will be found teeming with organisms of many kinds, among which *B. subtilis* will be prominent. Numbers of other species of *Bacillus* will also be found. In past decades little or no distinction was made between the various bacteria occurring in hay infusions, since all resemble each other very closely. They were grouped under the general term "hay bacillus." The term should be discarded, but is still used by some almost as a synonym for *B. subtilis* (Fig. 237).

Bacillus subtilis often forms long chains of bacilli sometimes called "streptobacilli." Since the bacilli are motile, such chains swim with a writhing motion. Individual cells or pairs of cells also occur. Due to avidity for oxygen, *B. subtilis* and many other species of *Bacillus* grow in a scum or pellicle at the surface of fluid media.

Due to its active attack on organic nitrogenous compounds, its cultures smell of ammonia. It grows well on any ordinary organic

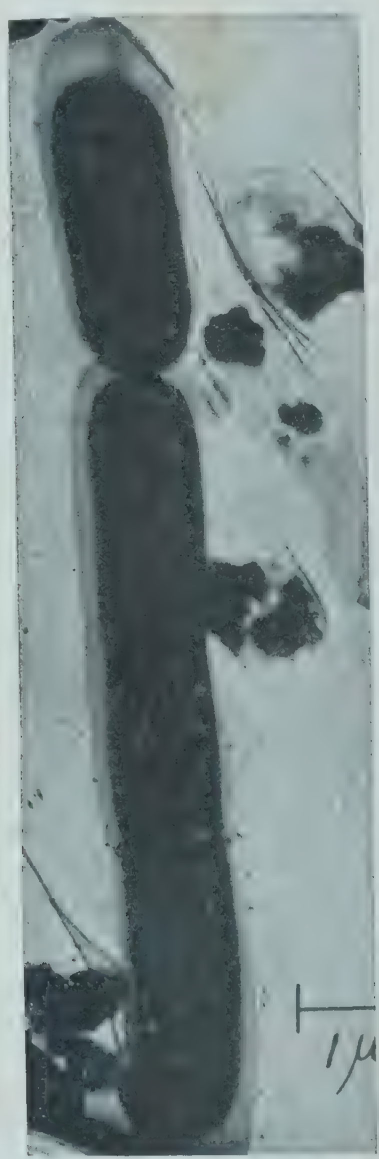

Fig. 237. Electron microscope picture of *Bacillus subtilis* (× 12,200). Note the continuous cell wall with thin column of cytoplasm extending from one cell to the other. Note the place in the lower cell where there is a slight indentation and thickening suggestive of a place where cell division may occur. Compare with Figure 235. (Mudd, Polevitsky, Anderson and Chambers, J. Bact., vol. 42.)

medium. On slants of potato it grows luxuriantly, with a yellowish or pink color and a warty or vesiculated appearance. The appearance of growth on potato is a means of differentiating between closely related species of spore-bearing, aerobic bacilli, but is subject to much variation.

Pathogenic Properties of Bacillus Subtilis. *B. subtilis,* and organisms closely related to it, have been isolated occasionally from pathological processes in human beings.

What is B. subtilis? For some years an interesting controversy has been maintained as to the true identity of *B. subtilis.* A culture originally described by Cohn in 1872, and maintained in a laboratory at Marburg, Germany, was for years held to be the true *B. subtilis.* It is characterized especially by small cells. Another strain, isolated by Lawrence and Ford from milk, in Baltimore, and characterized by larger-sized cells, has also been maintained as the true *B. subtilis.* Studies by Lamanna, Knaysi, and others show that the Marburg strain is identical with *Bacillus vulgatus,* while the Lawrence and Ford strain is the true *B. subtilis.*[11, 12] This controversy suggests the necessity for very carefully describing organisms of this group, and questioning the identity of any species unless so described.

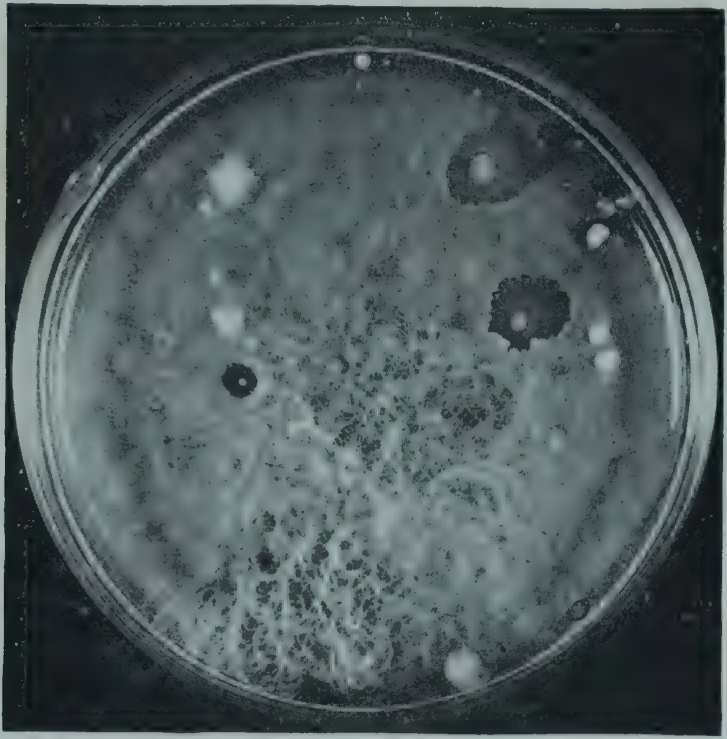

Fig. 238. Plate showing curious and typical curled growth of Bacillus mycoides on agar surface. Note also three colonies of contaminants; each showing a sterile zone about it, indicating production of antibiotic substance (Fleming's phenomenon). (Courtesy of Dr. Albert Kelner.)

BACILLUS CEREUS, B. MYCOIDES, B. VULGATUS, B. MESENTERICUS

These organisms are very much like *B. subtilis.* All are "hay bacilli" and all belong to the group of mesophilic species. *B. cereus* forms a spreading, grayish growth on agar, while *B. mycoides* forms very characteristic, moldlike, or nebula-like colonies on agar plates (Fig. 238). On potato it produces a whitish, granular growth which becomes brownish later.

Bacillus vulgatus forms a gummy slime and sometimes causes bread to seem "ropy" by growing in it during damp, warm weather, especially if the bread was made with flour containing many spores of this organism. Its growth on potato is thick, pinkish and wrinkled and turns brown.

Bacillus mesentericus is a slime-forming species also; its colonies are sticky and mucoid. On potato it forms a moist-looking, wrinkled scum which turns a dirty brown color later. It may be that *B. vulgatus* and *B. mesentericus* are identical organisms. This is interesting in view of the note on identity of *B. subtilis,* above. The close relation of these organisms is further seen in the fact that *B. mycoides* and *B. cereus* produce the enzyme lecithinase, which is not produced by other species of *Bacillus.*[12a]

BACILLUS PALUSTRIS

This species was an obscure soil organism but has come into prominence because, as shown by Sickles and Shaw[6, 13] it produces enzymes which attack the capsules of pneumococci. A score or more of very similar species have found to produce similar enzymes and also antibacterial substances, such as the important drug subtilin, bacitracin, polymyxin, gramicidin, tyrothricin,[14, 15] etc. Such drugs are discussed elsewhere.

"Milky White" Disease. Two other species, called *B. popilliae* and *B. lentimorbus* are now used to combat Japanese beetles. The organisms grow in the "blood" of the larvae and cause the disease commonly known as "milky white" disease. The larval juices are dried and ground and mixed with chalk dust or other powder. This is applied to the soil as a spray or dust.[16] Recent studies have been reported on the best sorts of media to induce growth and sporulation of the organisms.[17] It is earnestly hoped that the method will help control the beetles![18]

BACILLUS ROTANS[19] AND B. ALVEI[20]

These species are of especial interest because they produce colonies which move. Instead of merely spreading outward, the whole colonies move in a rotatory manner and actually travel from place to place. The individual bacilli composing the colonies are also motile. These motile colonies are discussed in the chapter on Myxobacteriales (page 428).

Foulbrood. *Bacillus alvei* is of especial importance because it is one of several organisms which cause, or are associated with, a disease (foulbrood) of bees which results in great losses to bee-keepers annually. There are several forms of the disease. American foulbrood is caused by a related organism, *B. larvae,* while *B. alvei* causes European foulbrood. Certain streptococci (*S. apis*) also appear to cause the disease. The larvae of bees contain the infecting organisms in large numbers. There may be another disease, "parafoulbrood," due to a separate organism, *B. para-alvei.*[21]

LACTOSE-FERMENTING BACILLI

Certain species of sporeforming aerobic bacilli are of importance as sources of error in the examination of water for sewage pollution (see page 581). They ferment lactose with gas formation and grow aerobically, and may be mistaken for *E. coli* or *A. aerogenes.*[22]

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Chapter 29

ANAEROBIOSIS. THE GENUS CLOSTRIDIUM

Anaerobiosis. Prior to the investigations on "diseases" of beer and wine carried out by Pasteur about 1860, it had been supposed that, in order to live, every creature was obliged to have air. The discovery of oxygen by Priestley in 1774 and the observations of Lavoisier on the nature of combustion led to the conclusion that free oxygen is necessary to life. In 1861, however, Pasteur proved that certain yeasts and sporebearing rods could multiply in the absence of air (or free oxygen). He devised the term "anaerobiosis" to describe this "life without air." This was one of the momentous discoveries in biological science. Subsequent studies in the physiology of cells living in situations devoid of free oxygen revolutionized ideas of cell physiology and metabolism.

Since Pasteur's researches a very considerable number of organisms capable of living without air have been discovered. Some are facultative anaerobes, *i.e.,* organisms capable of anaerobiosis or aerobiosis; some are strict or obligate anaerobes like *Bacteroides, Clostridium,* etc., incapable of life in contact with free oxygen. The mechanisms of anaerobic life have already been discussed (see page 292).

For some years the systematic study of strictly anaerobic bacteria was not as extensive as studies of aerobic and facultative organisms largely because of technical difficulties involved in producing environments free from uncombined oxygen and maintaining them so. Another great stumbling block in the investigation of these organisms was the difficulty of isolating pure cultures. Many anaerobic bacteria are of medical importance,[1] as well as industrial and agricultural value, so that good methods for study in the laboratory, especially in pure cultures, are essential.

Methods for the Cultivation of Anaerobic Bacteria. For many years anaerobic technics have been based on the assumption that free oxygen (air) must be removed from cultures of obligately anaerobic organisms and absolutely excluded from them during the period of growth. In the sense that free oxygen must be kept from accumulating in the immediate vicinity of the organisms this is correct. However, knowledge of the relationship between anaerobes and their gaseous environment has advanced to a point where it is possible to cultivate these organisms in ordinary culture vessels freely exposed to air and to eliminate to a great extent the use of the relatively clumsy and elaborate anaerobic apparatuses hitherto thought indispensable to investigations in this field. On the other hand, under some circumstances the apparatuses of the past are still useful. A description of some of them is given here. An excellent summary of these and other methods has been given by McClung.[2]

In spite of the fact that many types of anaerobic device exist, only one fundamental purpose is involved. This is the removal of *free* oxygen from the *immediate environment* of the bacteria, or the maintenance of a *low oxidation-reduction potential.* For the reader not familiar with this term a brief discussion is given at this point.

Oxidation-Reduction Potentials. A substance which readily takes up electrons is a good *oxidizing agent; i.e.,* is *readily reduced;* and, conversely, a good reducing agent is one which readily gives up electrons.

It is well known that an electrical current consists of a transfer of electrons between two points which are at different electrical potentials. If we thrust a platinum electrode into a vessel containing a strong oxidizing solution (ready to take up electrons) and connect it, by means of a good conductor of electrons (*e.g.,* a tube filled with agar containing an electrolyte, KCl) with a vessel containing a strong reducing solution (ready to give off electrons), a transfer of electrons must necessarily occur when the circuit between the vessels is completed. If an instrument (voltmeter or potentiometer) be introduced into the circuit, the difference in potential between the two solutions may be measured.

If, by means of a potentiometer, we find the potential difference between the two halves of this system and arbitrarily state the value of one as zero, then we have merely to make the voltmeter reading to find the other.

The "known" portion (which is called a "reference half-cell") is frequently prepared by bubbling hydrogen at one atmosphere of pressure through a N/1 solution of HCl (in which the HCl is completely dissociated); *i.e.,* 1 gm. H ions per liter (*p*H O). The fluid then contains H_2 gas and H^+ ions. Since each ion tends to lose an electron, and each atom to take an electron, a state of electron unbalance exists between the H gas and the H ions. Electrons tend to be transferred from the former to the latter:

$$\underset{\textit{Gas}}{H_2^{(2\ \text{electrons})}} \qquad \rightleftarrows \qquad \underset{\textit{Ion}}{2H^+ + 2\ \text{electrons}}$$

or there exists an oxidizing-reducing system which remains at a definite state of electron unbalance (or electrical potential). This cannot be measured directly by instruments but may be used as a "point of reference" for comparison with "unknowns."

If the "unknown" half-cell consists of a mixture of $FeCl_2$ and $FeCl_3$ (Fe^{++} + Fe^{+++}) then a similar state of unbalance exists, since the cation of higher valence (Fe^{+++}) has only 2 electrons while the cation of lower valence (Fe^{++}) has three. The $FeCl_3$ acts as an oxidizing agent, the $FeCl_2$ as a reducing agent because the former tends to take electrons from the latter, and the system is therefore an oxidizing-reducing system. Obviously, the greater the proportion of oxidizing agent, the stronger will the oxidizing tendency be, or the greater the tendency to take up electrons, or the higher the potential. The oxidation-reduction potential of a solution, therefore, results from an electron unbalance within it, and the degree of this unbalance depends largely on the ratio: $\frac{\text{oxidizing agent*}}{\text{reducing agent}}$.

Anaerobic conditions (*i.e.,* low oxidation-reduction potentials) in culture media may be brought about by three main classes of procedure.

(1) *Chemical.* (*a*) Cultures may be enclosed in an airtight vessel with sticks of phosphorus or with a freshly made mixture of potassium hydroxide and pyrogallol. These substances absorb large amounts of oxygen and leave mainly the inert gas, nitrogen, and a partial vacuum.

(*b*) The combustion of small amounts of alcohol or the burning of a small candle in a closed jar will also use up much of the free oxygen, replacing the air with a mixture of nitrogen and carbon dioxide. Combustion ceases when the carbon dioxide content approximates 10 per cent. This method results in only partially anaerobic conditions and is widely used to increase the carbon dioxide content of the atmosphere, a condition favorable to many organisms, both aerobic and facultative.

(*c*) A means of *absolute anaerobiosis* is to allow a fine stream of hydrogen to enter a closed vessel, impinging, as it enters, on a small mass of some catalytic agent which causes it to combine with the free

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* It must be pointed out here that two oxidizing-reducing solutions may each contain equal mixtures of bivalent and univalent cations as, for example: solution 1 = cupric (++) chloride and cuprous (+) chloride; solution 2 = stannic (++) chloride and stannous (+) chloride. Solution 1 will have a higher potential than solution 2. This results from a different innate tendency of the 2 metals to take up electrons which determines which solution will be at the higher potential.

When the hydrogen half-cell described above is used as a "reference cell" to complete the system in measuring the oxidization-reduction potential of any solution, the latter electrode potential (E) is expressed in terms referred to the potential of the hydrogen electrode (H) and is therefore called the Eh (oxidation-reduction potential *referred to hydrogen*):

$$\text{Eh} = \text{E}_{\text{ref.}} - \text{E}_{\text{meas.}}$$

It has been shown that the Eh of any oxidation-reduction system is directly affected by temperature, *p*H and the gas constant as well as by the ratio of oxidant to reductant. The relations of these factors have been computed on a thermodynamic basis and verified experimentally and have been expressed in the formula.

$$\text{Eh} = \text{E}_{\text{o}}^{1} + \frac{\text{RT}}{\text{nF}} . \ln . \frac{\text{ox}}{\text{red}} .\dagger$$

† R = gas constant; T = absolute temperature; F = faradays of electricity required to transfer 1 electron from the point of low potential to the point of higher potential; ln = normal logarithm of the ratio $\frac{\text{ox}}{\text{red}}$. (Where *ox* and *red* are equal, then $\frac{\text{ox}}{\text{red}} = 1$. Since ln 1 = 0, then Eh = Eo.)

oxygen, forming water. A catalyst commonly used is "platinized asbestos" (finely divided platinum deposited on asbestos fibers). This acts very rapidly only when heated. In some types of modern apparatus, the necessary heat is applied to the catalyst by means of an electric current passing through a resistance wire surrounding the mass of catalyst. In the modification devised by Brewer,[3] danger of explosion due to sparks is eliminated by enclosing the heating element in a gas-tight tube inside the catalytic mass (Fig. 239). A drying agent is enclosed in the vessel to absorb the water that is formed.

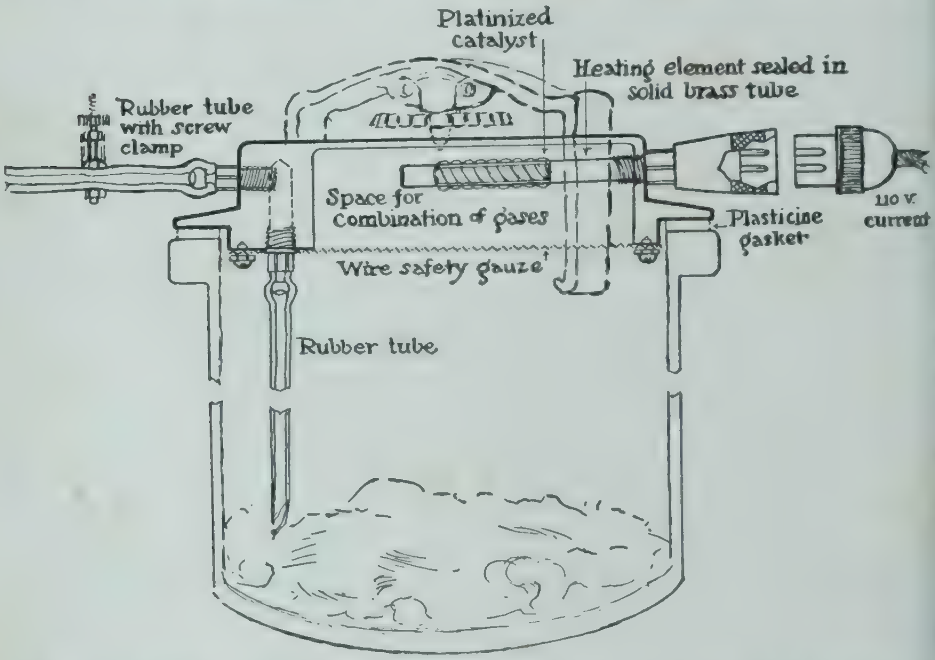
Platinized catalyst
Heating element sealed in solid brass tube
Rubber tube with screw clamp
Space for combination of gases
Plasticine gasket
110 v. current
Wire safety gauze
Rubber tube

Fig. 239. Brewer Anaerobe Jar. Note the tube for admission of hydrogen, the enclosed heating element and the platinized asbestos wrapped around it. The upper portion of the jar, where the heating element is located, is separated from the rest of the space by a wire gauge screen, a safeguard based on the principle of the Davey miner's lamp. (Brewer, J. Lab. and Clin. Med., vol. 24, C. V. Mosby Co., publishers.)

(2) *Replacement Methods.* Another method of removing oxygen from the atmosphere of closed "anaerobic jars" is simply to flush out all the air with a stream of some inert gas like hydrogen or nitrogen. This leaves an atmosphere of almost pure hydrogen or nitrogen and no vacuum.

(3) *Oxygen Exclusion Methods.* (*a*) A simple means of excluding oxygen from single culture tubes is to boil or autoclave them just before use, cooling rapidly in ice water (so that oxygen is not reabsorbed) before inoculation. The heat drives off the dissolved air. The tubes, if of broth, are then sealed by putting a layer of oil, petro-

leum jelly or melted agar, several centimeters thick, on the surface. If the organism is a gas-former, the gas may blow the petroleum jelly or agar plug out of the tube. This method is "messy" but effective if carefully manipulated. Withdrawal of material from such sealed tubes is awkward.

(*b*) We are indebted to Brewer[1] for a simple and effective means of obtaining pure cultures of strict anaerobes. This depends on chemical absorption of oxygen from air trapped in a very thin layer over the surface of special agar medium in a Petri dish. The cover of the dish is modified by having most of the central portion depressed so that it comes very close to the agar in the bottom part of the dish. A deeper annular depression near the rim actually comes into contact with the agar so that the central part of the agar surface is entirely sealed in, with a layer of air above it only about 1 mm. in thickness (see Fig. 240). The oxygen in this air is absorbed by sodium thioglycollate, or some similar compound having an affinity for oxygen, incorporated in the agar. By this means the O-R potential of the medium is held very low and even the most sensitive anaerobes will grow.

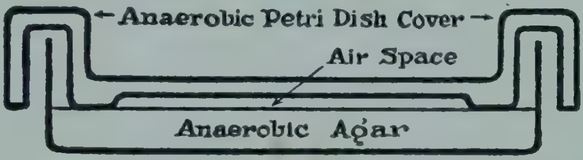

Fig. 240. Cross section showing Brewer anaerobic Petri dish cover in use.

(*c*) Deep tubes of dextrose infusion agar are also used to cultivate anaerobes. Infusion agar in tubes 8 to 10 cm. in depth is melted and cooled to about 50° C. The inoculum is put in and mixed thoroughly. The agar is then made to solidify rapidly in *cold* water and is incubated. Strict anaerobes will grow only in the depths and will not appear at all within a centimeter or more of the surface. Less strict anaerobes will grow in the depths and will also grow somewhat nearer to the surface, while facultative anaerobes will grow on the surface as well as in the depths. Organisms having a narrow zone of tolerance to both oxygen and strict anaerobiosis (*microaerophils*) may grow in a narrow zone some distance below the surface (Fig. 241). Such preparations are often spoken of as "shake tubes" because shaking is used to mix the agar and the inoculum.

Cultivation of Anaerobes in Media Freely Exposed to Air. Pure cultures of the strictest anaerobes may be obtained in ordinary media with no precautions as to exclusion of air, provided some substance be added to the media to maintain a low O-R potential.

For example, anaerobiosis in tubes of broth is satisfactory if the medium contains bits of chopped tissue, *e.g.*, Holman's "cooked meat medium"* or some modification of it. The tissue acts as a reducing agent. The meat also serves as pabulum for the bacteria. Most anaerobic bacteria grow well in cooked meat medium.[6]

As shown by Brewer,[6] the addition of sodium thioglycollate (0.1 per cent) or sodium formaldehyde sulfoxalate (0.1 per cent), or both, to dextrose broth or similar fluids adapts them to anaerobic requirements. The addition of 0.1 per cent agar creates a very slight viscosity which reduces aeration of the solution by convection currents. T'ung[7] has found that the use of 0.2 per cent cysteine hydrochloride is as effective, under most conditions, as the reagents mentioned above.

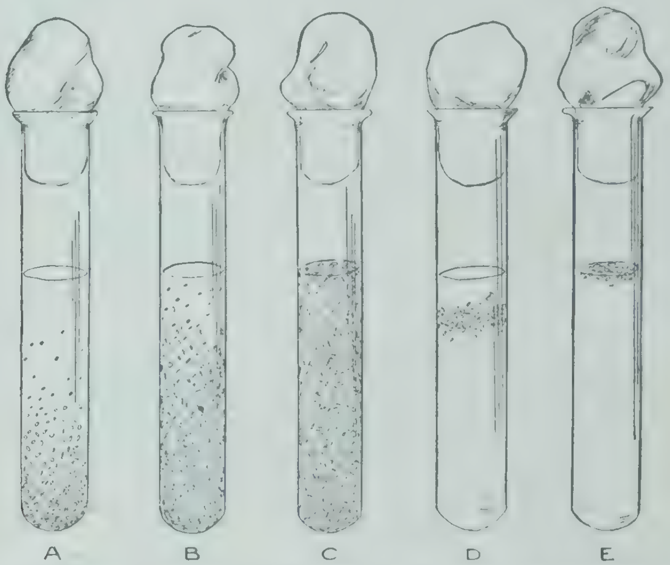

Fig. 241. Deep tubes of agar inoculated with bacteria of various oxygen relationships. *A*, Fairly strict anaerobe, like *Cl. botulinum; B*, less strict anaerobe, like *Cl. welchii; C*, facultative aerobe-anaerobe, like *Esch. coli; D*, micro-aerophilic organism like *Br. abortus; E*, strict aerobe, like *Pseudomonas fluorescens.*

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* **Cooked meat medium:** 250 gm. of fat- and fiber-free beef heart tissue are ground several times through a "fine" meat chopper. Sufficient distilled water is added to about double the volume of the chopped meat and the mixture is brought to a boil. The meat may be passed again through the chopper, replacing it afterward in the water in which it was heated.

Add 0.5 per cent peptone, 0.5 per cent sodium chloride and 0.1 per cent dextrose. Distribute in tubes so that they contain a column 8 to 10 cm. deep consisting of about 4 to 5 cm. of the chopped meat. They may be sterilized by autoclaving.

This medium is usually somewhat acid and may be adjusted in the usual manner but, due to the buffering action of the meat, will require several readjustments after being boiled.

In general, milk, infusion broth and infusion agar with blood, treated with the reducing reagents, are good culture media for anaerobic organisms, since these organisms require media rich in organic matter and having a *p*H of about 7.2. Chopped brain, fish or other tissues are also often used. The addition of dextrose provides a readily available source of energy which promotes the growth of nearly all anaerobes.

Reed and Orr and others have shown that an O-R potential of minus 0.2 volt is optimum for the *initiation* of growth by most species

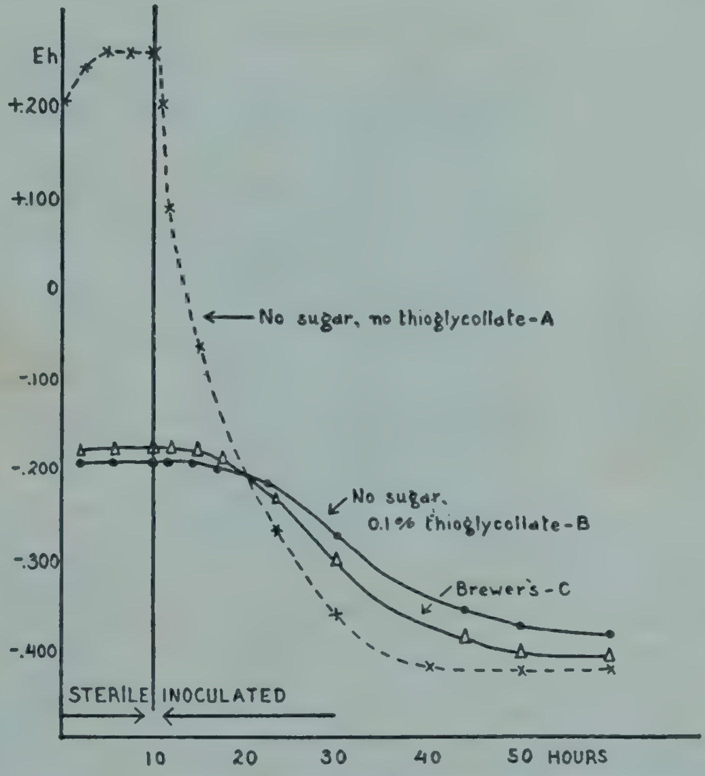

Fig. 242. Graphs indicating the O/R potential of growing cultures of *C. elchii* in peptone-agar medium with and without sodium thioglycollate and Brewer's medium. Ordinates represent Eh and abscissa time. (Reed, G. B., nd Orr, J. H., in J. Bact., vol. 45.)

f pathogenic clostridia. This is probably optimal for the nonpathogens s well. The effect of various substances added to media to maintain itially low O-R potentials are shown in Figure 242. Once growth has arted, the O-R potentials go much lower than this as the anaerobes se every available hydrogen acceptor in their respiratory processes.[8]

Purification of Anaerobe Cultures. *Separation of Aerobic and naerobic Sporeformers.* Cultures of sporeforming bacteria sometimes ecome contaminated with non-sporeforming bacteria. The culture ay be freed from the latter by heating. However, cultures of anaerobic sporeformers may also become contaminated with sporeforming aerobes. Under anaerobic conditions, anaerobes will form spores that are heat-resistant, while aerobic sporeformers do not produce spores in the absence of free oxygen. By cultivating the mixture under strictly anaerobic conditions, sporulation of the aerobic forms is inhibited. They may then be killed by heat.[9] McClung has described a very useful method for the isolation and purification of clostridia,[10] which will prove of value to students of the genus. The Brewer plate is also invaluable.

GENUS CLOSTRIDIUM

The bacteria belonging to this genus are all obligately anaerobic, gram-positive, spore-bearing rods. The group includes the organisms producing tetanus (lockjaw), gas gangrene, and botulism (food poisoning), but the majority of clostridia are harmless and helpful saprophytes. Many of them produce industrial fermentations of great value. Nearly all are motile, and all occur widely distributed in the soil. Some of them also live in the intestinal tract of man and animals. They are metabolically active and versatile.

TABLE VII

SOME METABOLIC PROPERTIES OF COMMON ANAEROBIC, SPORE-BEARING RODS*

Carbohydrates Attacked	Organisms Attacking		
	Gelatin only	Casein and gelatin	Casein, gelatin, serum, egg, fibrin
Starch, saccharose, lactose and dextrose	Cl. tertium† Cl. chauvoei Cl. butyricum‡ Cl. fallax†	Cl. perfringens	Cl. putrificum
Dextrose, saccharose *and* lactose	Some soil species		
Dextrose, and saccharose *or* lactose	Cl, septicum and Cl. novyi		
Dextrose only			Cl. botulinum
None	Cl. tetani		Cl. histolyticum

* For explanation of the arrangement see text.

† Does not attack gelatin.

‡ Not a pathogenic species. Closely related industrial species may be included here.

Many of the pathogenic anaerobic sporeforming rods seem to be endowed with the powers of fermentation and proteolysis in a compensatory manner. That is, those which are highly proteolytic are less actively fermentative and vice versa. There are, however, as in all biological groupings, no absolute lines of demarcation. Table VII, adapted from Brown,[11] shows important biochemical properties of some of the common clostridia and gives their names.

In this table the organisms are listed vertically in the order of chemical complexity of carbohydrates which they can attack. They are arranged horizontally according to the chemical complexity of protein which they hydrolyze. Thus, *Clostridium chauvoei* (the cause of a gangrene-like disease of cattle, called "blackleg") attacks all of the carbohydrates listed but can hydrolyze only the incomplete protein, gelatin. *Cl. chauvoei, Cl. perfringens, Cl. septicum,* and any other organisms having similar properties, are said to be predominantly "saccharolytic" organisms, while those like *Cl. tetani* and *Cl. histolyticum* are predominantly proteolytic. Other clostridia are intermediate in these respects. Other tests used in identifying clostridia are fermentations of various carbohydrates, proteolyses, and certain special tests such as H_2S production, hemolysin production, nitrite reduction, etc. (see Table VIII).

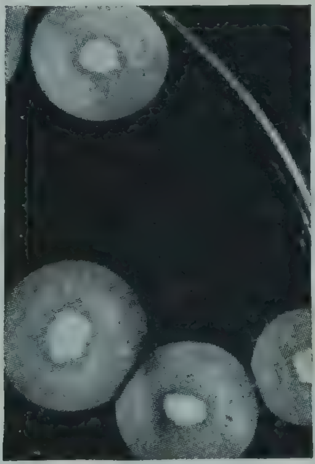

Fig. 243. *Clostridium novyi* type A showing the egg-yolk precipitation reaction around colonies on yolk-agar. (McClung, L. S., Toabe, B., in J. Bact., vol. 53.)

A peculiar reaction diagnostic of certain organisms of the group is the Nagler reaction. This has been studied in detail by McClung and his associates. A medium is prepared containing peptone, mineral supplement, glucose and agar. To this is added egg yolk and plates are poured. The solid medium is inoculated with pure cultures of the organisms to be tested. A positive reaction consists of a zone of opaque precipitate around the colony and a pearly "luster area" over both colony and zone. It is of particular interest that the reaction is inhibited if antitoxin corresponding to the organism being tested be incorporated in the agar. The effect is apparently due to certain toxins of the organisms. Specific reactions are given by *Cl. perfringens, Cl. novyi, Cl. botulinum* of various types, and some other organisms. *Cl. tetani* does not give the reaction.[12] Colmer has shown that precipitation of the phospholipid fraction of the egg yolk may be brought about by lecithinase of certain species of *Bacillus*. Whether this is the mechanism of the McClung reaction is not certain.[12a]

CLOSTRIDIUM BUTYRICUM

This is among the earliest species of Clostridium to be studied and is the type species of the genus (Fig. 244). It represents the group of industrially important clostridia, all much alike. These organisms are widely distributed in the soil. All have the power of attacking carbohydrates, with the production of *butyl alcohol* and *butyric acid* and other organic compounds of industrial value. Their differentiation is often difficult. In general they are actively motile rods having oval, excentric spores which swell the sporangium. Some of them are of great importance in the manufacture of butyl alcohol which is of value in many industrial processes. Organisms of this group of butyl alcohol and butyric acid formers are often spoken of under the general term of "butyric-acid bacteria" or "butyl-alcohol bacteria." Some varieties, *e.g.*, one called *Cl. acetobutylicum* and others called *Cl. butylicum* and *Cl. pastorianum*, also produce *amyl, ethyl,* and *propyl* alcohols and *acetic, formic,* and *lactic* acids, *acetone*, carbon dioxide and hydrogen. The products of fermentation depend on the variety of

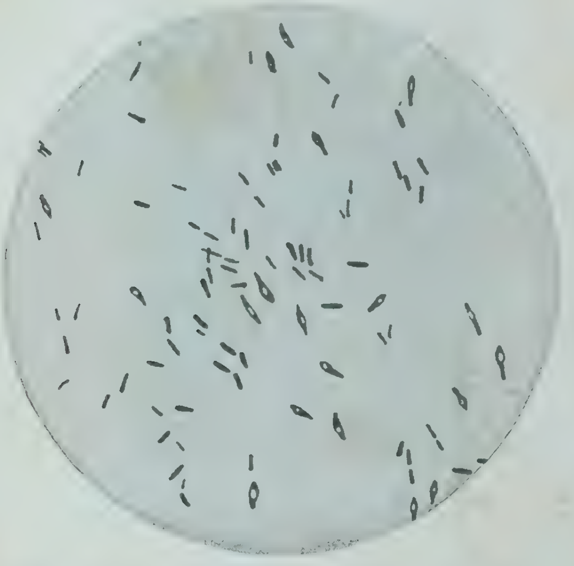

Fig. 244. *Clostridium butyricum* (× 900).

Clostridium used and the conditions of the fermentation, *i.e.,* nutrient, *p*H, temperature, substrate, etc. *Cl. acetobutylicum* produces large amounts of acetone and butanol from starchy substrates (see section on Industrial Applications of Bacteriology, page 680). Some varieties of the organisms synthesize and store up glycogen, starch and similar substances in the form of granules within the cell. This gave rise to the older generic name *Granulobacter* which expresses these properties. Spray has shown that the power to synthesize "granulose," a starch-like polysaccharide, from glucose is especially distinctive of two of the "butyric" organisms: *Cl. pastorianum* and *Cl. beijerinckii.*[12b]

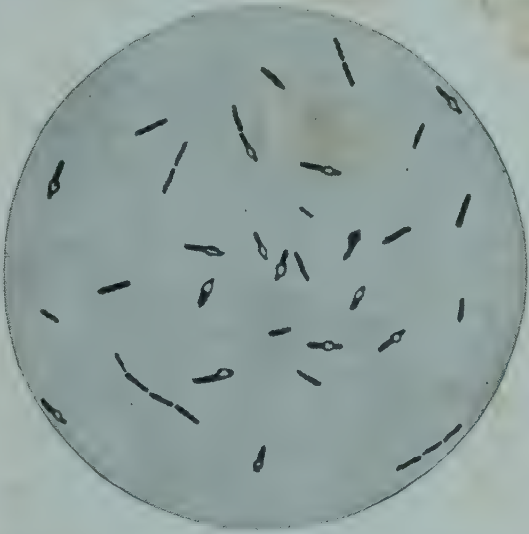

Fig. 245. *Clostridium felsineum* (× 900).

Anaerobic Nitrogen Fixation. An interesting property of some of these organisms (notably *Cl. butyricum*) is the power to fix atmospheric nitrogen. That is, they do not require nitrogen combined in the form of ammonia, nitrates, amino acids, etc., but possess the power to cause free nitrogen of the air to combine in the synthesis of their protoplasm. This valuable property is found in very few species of bacteria but is of the utmost importance in soil fertility (see nitrogen cycle, page 609). They grow well in poorly aerated soils, where they are active in hydrolyzing cellulose and fermenting the products of this decomposition (Chapter 35). They also grow in good agricultural soils.

CLOSTRIDIUM FELSINEUM

This species is closely related to *Cl. butyricum* but is proteolytic. It is also highly fermentative and has the power of digesting the pectin or cement-like gum of plant tissues so that it is useful in the preparation of flax, facilitating separation of the bast fibers from the rest of the plant tissue (see retting of flax, page 695, Fig. 327).

PATHOGENIC CLOSTRIDIA

An important paradox is that although highly dangerous pathogenic organisms, *Clostridium botulinum, Cl. perfringens* and *Cl. tetani* are not *parasites* but *strict* saprophytes. They grow only on dead matter and cannot invade live tissue. They are all commonly found in the soil, the latter two species especially in horse manure. The spores, consequently, are widespread in street dirt and manured lands. Morphologically and culturally all are much like the butyric-acid and similar organisms, but tend rather less toward fermentation and more toward proteolysis, although no sharp line of demarcation between the two groups may be drawn.

CLOSTRIDIUM TETANI AND "LOCKJAW"

Clostridium tetani is one of the strictest anaerobes (most sensitive to air). It is cultivable on blood-infusion agar plates or slants only in an atmosphere of inert gas like hydrogen or nitrogen. It grows well on Brewer plates and in broth containing 0.2 per cent cysteine hydrochloride, as well as in cooked meat medium. It ferments no carbohydrates, nor is it very active in attacking proteins. Morphologically, the organism is usually a slender rod (0.5μ by 4 to 8μ). It bears a spherical spore at the very tip end (terminal) of the rod. The round, terminal spore gives to the organism what has been called a "drumstick" appearance (Fig. 246). It is thus very different in appearance from *Cl. perfringens, Cl. sporogenes,* etc., which produce oval spores located centrally or excentrically.

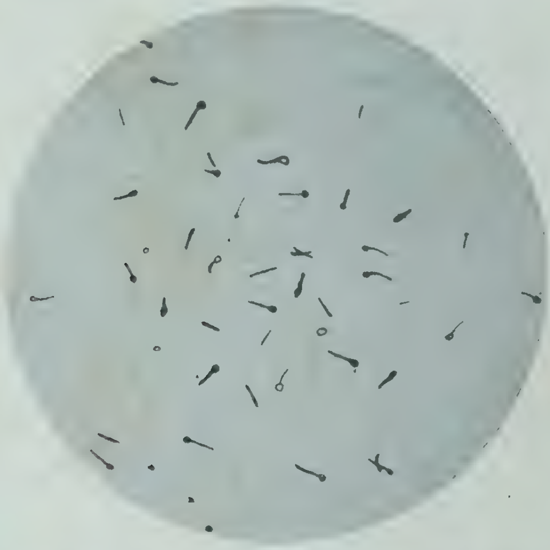

Fig. 246. *Clostridium tetani* (× 900). Note the terminal spores.

Cl. tetani gives off a very potent exotoxin. Tetanus toxin is particularly active in the motor nerve centers, irritating them so that the muscles connected with them are thrown into a state of violent and continuous contraction (tetanic convulsion or tetanus). By injecting the toxin in small amounts into horses, a potent antitoxin may be prepared which is very valuable in the treatment and prevention of tetanus. This use of antitoxin is a classical example of passive immunity and was the first to be discovered (von Behring and Frankel, 1890). Once the toxin has combined with and affected the nerves, however, antitoxin is almost useless.

Tetanus organisms gain entrance to the body with dirt or dirty objects when these are forced into the tissues as in gunshot or shrapnel wounds or various accidental means. Under such circumstances some tissue is killed locally by the mechanical injury and, in deep wounds, the low O-R potentials favor growth of anaerobic bacteria. The organism *cannot* invade the *body,* but grows as a saprophyte on the dead tissue in the wound, liberating its deadly toxin which is absorbed by the blood or nerves or both.

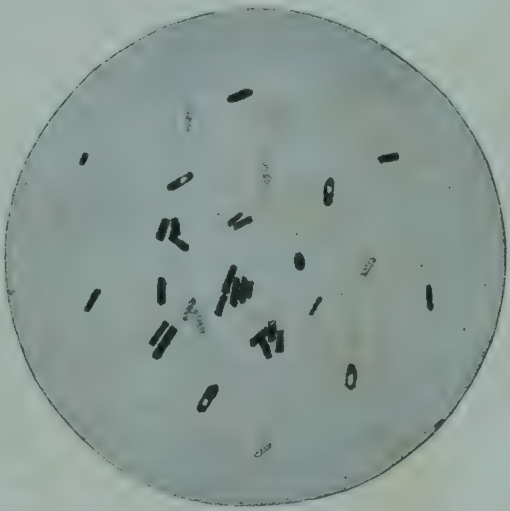

Fig. 247. *Clostridium perfringens.* Cultivated in cooked meat medium (× 900).

Tetanus toxin is one of the most potent poisons known. It requires only about 0.00025 gm. of tetanus toxin to kill a man while it requires twenty times as much cobra venom and about one hundred and fifty times as much strychnine to do the same.

Tetanus Immunization. Alum-precipitated and fluid toxoid, in all respects analogous to diphtheria toxoid (see page 331), are useful in producing active immunity to tetanus. Protection depends particularly on the action of a primary stimulus (see page 330) consisting of a single dose of tetanus toxoid. The body cells respond quickly with the production of antitoxin, to a secondary stimulus resulting from entrance of tetanus toxin to the body as the result of a wound. A dose of toxoid in previously immunized persons is therefore often used as prophylaxis in dealing with any fresh wound, unless tetanus is imminent, when passive prophylactic antitoxin may be used. The same principles apply in diphtheria (see page 745). Combined diphtheria and tetanus immunization employing mixtures of the two toxoids has been found entirely practicable.[13, 14] The army's experience with fluid toxoid has been very favorable.[15]

TABLE VIII

BIOCHEMICAL REACTIONS OF GAS GANGRENE CLOSTRIDIA*

Species	Milk	Dextrose	Maltose	Lactose	Salicin	Sucrose	Hydrogen sulfide	Gelatin liquefaction	Nitrate reduction	Indol	Milk agar digestion	Exotoxin
Cl. welchii (*perfringens*)	Stormy	+	+	+	—	+	+	+	+	—	—	+
Cl. butyricum (group)	Stormy	+	+	+	+	+	+	+	+	—	—	—
Cl. multifermentans	Stormy	+	+	+	+	+	—	—	+	—	—	—
Cl. aerofœtidum	Stormy	+	+	+	+	—	+	+	+	—	—	—
Cl. tertium	Acid	+	+	+	+	+	+	—	+	—	—	—
Cl. fallax	Acid	+	+	+	+	+	+	—	+	—	—	+
Cl. paraputrificum	Acid	+	+	+	+	+	±	—	—	—	—	—
Cl. carnis	Acid	+	+	+	+	+	—	—	—	—	—	+
Cl. chauvœi	Acid	+	+	+	—	+	+	+	+	—	—	+
Cl. septicum	Acid	+	+	+	+	—	+	+	+	—	—	+
Cl. sphenoides	Acid	+	+	+	+	—	+	—	—	+	—	—
Cl. novyi (*œdematiens*)	Digested	+	+	—	—	—	+	+	—	—	±	+
Cl. bifermentans	Digested	+	+	—	—	—	+	+	—	+	+	—
Cl. sordellii	Digested	+	+	—	—	—	+	+	—	+	+	+
Cl. sporogenes	Digested	+	+	—	—	—	+	+	—	—	+	—
Cl. histolyticum	Digested	—	—	—	—	—	+	+	—	—	+	+
Cl. tetanomorphum	No change	+	+	—	—	—	±	—	—	±	—	—
Cl. difficile	No change	+	—	—	+	—	—	—	—	—	—	+
Cl. capitovalis	No change	+	—	—	—	—	±	±	—	+	—	—
Cl. cochlearium	No change	—	—	—	—	—	—	—	—	—	—	—
Cl. tetani	No change	—	—	—	—	—	+	±	—	—	—	+

* Reed and Orr, J. Med. Sciences, *206*.

CLOSTRIDIUM PERFRINGENS (THE "GAS BACILLUS")

Cl. perfringens is a rather short, thick rod with rounded ends. It unimportant exceptions, it is *the only nonmotile species in the genus.* It may be pointed out here that, in attempting to determine motility of anaerobic bacteria, care must be taken not to expose the hanging drop to the air for more than a few seconds as motility is destroyed by access of free oxygen. *Cl. perfringens* is a typical anaerobe, although not so intolerant of free oxygen as some other members of the group and is a constant inhabitant of the soil and of the intestine.

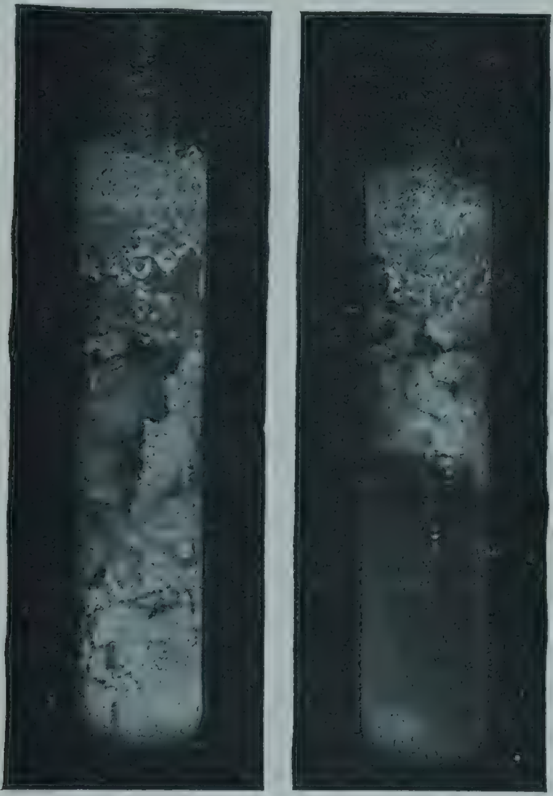

Fig. 248. Tube of milk inoculated with *Clostridium perfringens,* showing "stormy fermentation." (N. MacL. Harris prep.)

Its constant presence in feces has led at times to its consideration as an indicator of human fecal pollution when found in water. There are, however, serious objections to this. For example, it occurs also in soil and in animals and outlasts for years, by means of its spores, any danger of infection by organisms of the typhoid group. It gives falsely positive presumptive tests in bacteriological water examinations (see page 581) due to its ability to form gas from lactose under the partially anaerobic conditions in the depths of the fermentation tubes used for bacteriological water analysis. Because of its pronounced gas-forming powers it is often called the "gas-bacillus."

"Stormy Fermentation." One of its most characteristic cultural reactions is its power to produce a condition in milk called "stormy fermentation." If a column of milk 10 cm. deep in a culture tube be inoculated with *Clostridium perfringens* and incubated, the lactose is quickly fermented with the formation of sufficient acid to coagulate the casein. Gas (mainly hydrogen) is then formed in large amounts and the clot of casein is rent asunder, giving the appearance of a very turbulent or "stormy" reaction (Fig. 248). Any organism producing sufficient acid to form a solid clot, followed by abundant gas production, could give the same reaction. There are, however, only a few such organisms.[16] Four are listed in Table VIII.

Cl. perfringens will grow well on a purely synthetic medium containing, in addition to the usual mineral salts, 19 amino acids, 5 vitamins, glucose and adenine. Since it is utterly dependent on 14 of the amino acids, it can be used in the detection of these substances, as well as in their measurement (see Chapter 39).[16a, 16b]

THE GAS GANGRENE ORGANISMS

Cl. perfringens, being common in the soil, always accompanies *Cl. tetani* in wounds and, like the latter organism growing saprophytically only on dead tissue, gives off a toxin which is guarded against by prophylactic injections of antitoxic serum into wounded persons. These sera usually contain antibodies against all of the gas-gangrene group which is discussed below.

Gas Gangrene. In dirty wounds, in addition to *Clostridium perfringens* and *Cl. tetani,* there are nearly always present several other rather similar species of saprophytic clostridia of the soil such as *Cl. novyi, Cl. histolyticum,* etc. Some of these are able to digest dead tissue rapidly, others produce toxins, hemolysins,* etc.

In general, the combined growth of *Cl. novyi* or *Cl. perfringens,* and other gas gangrene organisms of a similar nature of which there are some twenty species, in dirty wounds such as crushed limbs, shell wounds, nail punctures, etc., where there is much dead tissue, if unchecked, produces a rapidly fatal condition known as *gas gangrene. Cl. perfringens* and *Cl. novyi,* growing in the dead tissues, produce large amounts of gas. The gas bubbles in the tissues expand and press upon the surrounding live tissues, shutting off their blood supply. The tissues, deprived of their blood supply, die. The highly proteolytic *Cl. histolyticum* and similar species then actively digest this dead, gangrenous tissue and the surrounding parts are thus further invaded by the gas gangrene group which extends the process very rapidly. The toxin given off by *Cl. perfringens* and other species aids in weakening the patient and, without treatment or amputation, death ensues very rapidly.

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* Toxin-like substances which destroy red blood corpuscles.

It must not be supposed that clostridia are the only flora of wounds. Wound bacteriology is very complex. In addition to clostridia, hemolytic streptococci, micrococci, and many other organisms are found, some capable of causing "blood poisoning" and toxemia (absorption of toxin into the blood). While the methods for anaerobic cultivation described here are adequate for wound bacteriology, much skill and experience as well as the concurrent use of aerobic methods are required. The bacteriological and clinical aspects of gas gangrene are well summarized by Reed and Orr[16] and by Rustigian and Cipriani.[17]

Dirty wounds are best opened and cleansed of dirt and dead tissue and exposed to air and disinfectant solutions such as H_2O_2, etc. Penicillin, tyrothricin, and other antibiotics are used to advantage in the treatment of wounds.

Clostridium perfringens has also been implicated as the cause of gastro-enteritis or food poisoning by its growth in certain foods. This is more fully discussed in the section on foods.

CLOSTRIDIUM SEPTICUM

This is a longer, more slender organism than *Cl. perfringens* and often grows in long wavy filaments. It produces oval, central spores. It has well marked fermentative (saccharolytic) powers, but it also attacks proteins having a higher degree of chemical complexity than gelatin. It is the cause of a fatal disease (malignant edema) in man and animals, similar to gangrene but without gas formation. There is accumulation of much fluid (edema), and moderate toxemia. The organism often produces *septicemia* (pathogenic bacteria growing in the blood; "blood poisoning"). It is also associated with *Cl. perfringens* and numerous other soil anaerobes in *gas gangrene*. It is of historical interest because it was the first pathogenic anaerobe to be described (Pasteur, 1877) who named it Vibrion septique.

CLOSTRIDIUM BOTULINUM

This is one of the three organisms (*Staphylococcus, Salmonella, C. botulinum*) most frequently causing food poisoning. *Cl. botulinum* is a strict anaerobe and forms large, oval spores in a subterminal position, often giving the sporulating rod a shape that is said to resemble a snowshoe (Fig. 249). Like the other clostridia, it is widely distributed in the soil.

It is quite proteolytic and somewhat saccharolytic as well. It derives its name from the Latin word for sausage (*botulus*). It was given its name because it was first found in sausages which were the cause of fatal food poisoning. The interior of a sausage obviously presents an ideal place for the growth of anaerobes.

Clostridium botulinum is a dangerous pathogen because it causes a highly fatal type of food poisoning, known as botulism. Yet it never grows extensively in or on the human body.* It is discussed in detail in the chapter on food (page 652). Botulinus toxin has been produced pure in quantities for certain investigational purposes.[19]

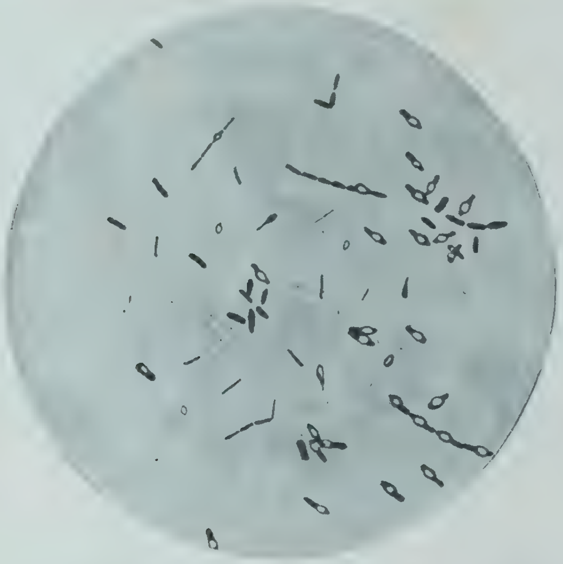

Fig. 249. *Clostridium botulinum* (× 900).

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* It has been found growing in wounds but apparently not enough toxin is formed in, or absorbed from, such situations to be harmful.[18]

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Chapter 30

THE GENUS CORYNEBACTERIUM

The name of this genus means "club-shaped bacterium," a term derived from club-like forms often formed by the very pleomorphic diphtheria bacillus (*Corynebacterium diphtheriae*) which is the type species. As a type species *C. diphtheriae* is somewhat misleading, as it differs from the majority of the other species of the genus in morphology, pathogenicity and certain distinctive growth characters. The species of *Corynebacterium* other than the diphtheria bacillus are often referred to collectively as "diphtheroids" although their resemblance to *C. diphtheriae* is in many instances very slight. It would be preferable to refer to them as corynebacteria.

The genus comprises gram-positive, non-sporeforming, non-encapsulated rods which grow aerobically or microaerophilically.[1] In general, they require complex organic nourishment, *p*H around 7.2 and temperatures ranging from about 30° to 40° C. depending on the species. Most are short and thick (2μ x 3 to 10μ) and of quite regular form. Only one or two species, as *C. xerose,* a common inhabitant of the conjunctival sac and throat, frequently form cells closely resembling *C. diphtheriae*. Others may occasionally do so. In general they are not active in attacking carbohydrates, proteins or fats.

Some authorities include motile, gram-positive, non-sporeforming aerobic rods in the genus, but it would seem preferable to place these in other genera. For example, the genus *Listeria* has been established for a motile corynebacterium-like organism widely distributed and thought to cause infectious mononucleosis or "glandular fever" in man and animals.[2] Other motile "diphtheroids" might well go into this genus.

A number of similar gram-positive rods now separated in the genera *Microbacterium, Butyribacterium* and *Propionibacter* are very closely related and very similar to corynebacteria in a number of respects. Excellent electronographs of *Microbacterium* have been prepared by Doetsch and Pelczar.[9]

Colonies. The colonies of corynebacteria on solid media, such as blood infusion agar, are generally white, or yellowish, opaque, round, and range in diameter from about 1 mm. to 3 or 4 mm. They are usually soft and butyrous (pasty) in consistency but some species form irregular and brittle colonies. Many species form brilliantly colored colonies.

Several species are pathogenic; notably *C. diphtheriae; C. hemolyticum* which caused infections in U. S. troops in Pacific islands during the war with Japan[3]; *C. ulcerogenes,* occasionally occurring in infections in man[4, 5]; *C. acnes,* occurring in acne pimples on the human skin;[6, 7] *C. pyogenes,* which is common in purulent lesions of cattle, swine and sheep; *C. equi,* causing pneumonia in foals; and *C. renale* which causes a necrotic disease of the urinary tract in cattle.[8] Some workers also include certain plant pathogens. A few usually harmless corynebacteria found in the normal nose or throat of man, as *C. flavidum,* may also occasionally cause disease in man and animals.

TABLE IX

USUAL FERMENTATION REACTIONS OF SOME COMMON CORYNEBACTERIA

Species	Lactose	Dextrose	Saccharose	Maltose	Gelatin and Milk*
Corynebacterium diphtheriae	−	+	−	+	−
Corynebacterium pseudodiphthericum	−	−	−	−	−
Corynebacterium xerose	±	+	+	+	−
Corynebacterium acnes	±	+	−	−	−
Corynebacterium pyogenes	+	+	+	+	+

* Proteolysis.

Habitat and Function. The saprophytic and nonpathogenic corynebacteria are widely distributed in soil, milk, dust, water, in the air, on the skin, in the wax of the ear, in the eye, nose and throat, and it is small wonder that they are frequently encountered as contaminants.

Except as scavengers aiding in decay, most of the nonpathogenic corynebacteria seem to be of relatively little importance from any standpoint except that of causing confusion to medical and veterinary bacteriologists by appearing as contaminants and being mistaken, sometimes, for the pathogenic species. This is particularly true of *C. xerose* and of *C. pseudodiphthericum* (also called "Hoffmann's bacillus" or C. hoffmannii) which occurs very frequently in the normal nose and throat. However, this organism may also act as a secondary invader causing serious complications in hemorrhagic septicemia of swine. Differentiations between some of the species of corynebacteria are made on the basis of fermentation reaction, pigment formation and morphology (Table IX).

Morphological Characters of Corynebacteria. Because of the practical importance of morphological studies in the diagnosis of diphtheria the appearances of corynebacteria are worthy of some detail in discussion.

Most of the diphtheroids are rather short, plump rods of a relatively *uniform length* and with a tendency to rounded ends. Metachromatic granules (volutin) occur but are not frequent, and club-shaped cells are not a prominent feature although sometimes seen. When the organisms are stained with certain solutions of toluidine blue and counterstained with iodine (Albert-Layburn stain)* the granules, when present often appear of a color different from the body of the rod and therefore are said to be *metachromatic* (Albert's stain, Ponder's stain). These two characters (granules and club-shaped cells) differ greatly among species and in different cultures of any given species. However, the general picture of diphtheroids is one of comparative uniformity in size and staining as compared with *C. diphtheriae* (Fig. 250).

Parallelism. The arrangement of the cells of diphtheroids is especially characteristic. When the cells divide, the cell membrane gives way on one side. A snapping movement then occurs and the two daughter cells swing around so as to lie side by side, like the two halves of a broken, green twig which are still held together by a shred of bark. This process, continued through several divisions, often results in numbers of cells lying side by side in what is called a "palisade arrangement" or parallelism (Fig. 250).

Corynebacterium diphtheriae differs from diphtheroids in that the cells are usually more slender, *vary greatly in length,* show much

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* *Formula for Albert-Layburn stain:*

Solution I—	Toluidin blue	0.15 gm.
	Malachite green	0.20 gm.
	Glacial acetic acid	1.00 ml.
	Alcohol 95%	2.00 ml.
	Distilled water	100.00 ml.
	Autoclave 15 lbs. 15 minutes.	
	Let stand 24 hours, filter.	
Solution II—	Iodine crystals	2.0 gm.
	Potassium iodide	3.0 gm.
	Distilled water	300.0 ml.

1. Fix smear by heat.
2. Flood with solution No. I for three to five minutes.
3. Wash in tap water.
4. Flood with solution No. II one minute.
5. Wash, blot dry and examine.

Granules dark blue; bacilli greenish.

larger and more numerous and definite metachromatic granules, and more club-shaped, spindle-shaped and other irregular forms. They often appear vacuolated or segmented, a feature commonly referred to as "barring." Palisade arrangement is much less definite in *C. diphtheriae* than in diphtheroids, irregular groupings suggestive of the characters used in Chinese printing being an especially useful diagnostic feature in addition to those mentioned above. Indeed, the mor-

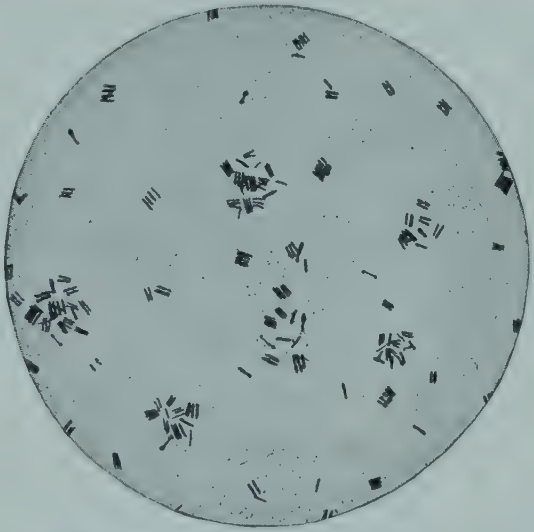

Fig. 250. Typical diphtheroid (*Corynebacterium pseudodiphthericum*) (× 900). Note regularity of length and arrangement, with only an occasional beaded and club-shaped cell. (Löffler's methylene blue stain.)

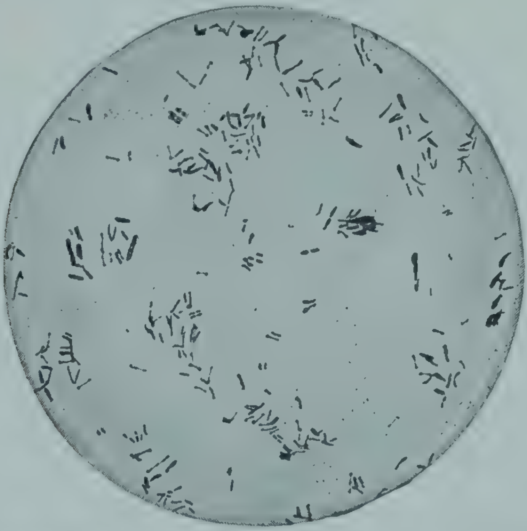

Fig. 251. *Corynebacterium diphtheriae,* cultivated on Löffler's medium after isolation in pure culture. Stained with Löffler's methylene blue (× 900).

phological features of *C. diphtheriae* are so striking that it is often the practice to confirm diagnoses of diphtheria on this basis alone (Fig. 251).

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Chapter 31

SPHERICAL BACTERIA. I. MICROCOCCACEAE

All of the cocci of major importance in industry, agriculture and medicine are included in the suborder *Eubacteriineae*. Here, except for the genus *Nitrosococcus* (see page 601), the most important are included in three well-defined groups, namely, Streptococceae, Micrococcaceae and Neisseriaceae.

All of these cocci are rounded or some modification of the spherical form (oval, elliptical, and so on). None forms spores and none is motile. Most of the cocci are aerobic or facultative. A few are strict anaerobes.

The cocci are divided into several subgroups on the basis of the form and arrangement of the cells (see Chapter 4). Several important genera are included in the family of Micrococcaceae, and we shall turn our attention first to these.

FAMILY MICROCOCCACEAE

General Characters. The organisms of this family are usually nearly spherical in form. Chain formation is not characteristic of these organisms, although occasionally as many as three or four cells will cling together in a row temporarily. Odd shaped clumps are the rule. Pairs of micrococci are frequently seen, especially in young broth cultures. Various other arrangements resulting from cell division in two or three planes are characteristic. The family includes some pathogenic species, notably *Micrococcus* (*Staphylococcus*) *aureus, Micrococcus* (*Staphylococcus*) *albus* and *Gaffkya tetragena* but most of the Micrococcaceae are saprophytes, harmless for man. Some cause serious diseases of animals, for example, mastitis of cattle.

All of the Micrococcaceae grow well aerobically at temperatures of from 30° to 40° C. on simple media, such as extract agar or peptone solution. A large majority form opaque, butyrous colonies colored with white, yellow or red pigments; various shades of red and orange are especially common among the saprophytic types. Micrococci have a marked tolerance for NaCl and can be isolated from mixed cultures on media containing 5 to 8 per cent salt, a concentration which inhibits growth of many other contaminating organisms.[1] All are gram-positive. They are widely distributed in soil, water, air, foods, human and animal skin and mucous membranes, etc.

Spatial Arrangements. The spatial relationships of the Micrococcaceae are best seen by observing broth cultures in hanging-drop preparations. Here the tetrads of the *Gaffkya** which divide in two planes at 90 degree angles, and the cubical packets of the *Sarcina*† which divide in three planes at 90 degree angles, are readily distinguished, as are frequent pairs and small, irregular clumps of *Micrococcus* (*Staphylococcus*) which divide in irregular manner. Large masses of mucilaginous capsular material may often be seen enclosing the tetrads of *Gaffkya.*

As shown by Foubert and Douglas, the distinctions between *Micrococcus, Sarcina* and *Gaffkya* on the basis of spatial arrangement, especially in the anaerobic species, is not always easy. They offer a system of biochemical differentiations for the anaerobes. It is of interest that these strict anaerobes produce catalase (see page 305).[1a]

Previously it was common practice to speak of the nonpathogenic micrococci as the genus *Micrococcus* and to reserve the generic term *Staphylococcus* for pathogenic species. The distinction between the micrococci and staphylococci is vague and present nomenclature includes both in the genus *Micrococcus.*

Role of the Micrococcaceae in Nature. Except for *M. aureus* and *M. albus* and *Gaffkya tetragena,* which are pathogenic, the Micrococcaceae as a family are of importance (as far as is known at present) mainly as scavengers. They are widely distributed in milk, soil, dust and water, especially where putrefaction is in progress. They are among the commonest contaminants in laboratory cultures. Many digest proteins like gelatin and casein and attack various carbohydrates and other organic substances. A few species (*M. cereus, M. perflavus, M. caseolyticus*) are of commercial importance in the ripening and flavoring of cheese since they attack casein and lactose with the production of aromatic substances having pleasing flavors. Some produce various undesirable conditions such as "ropy milk" (*M. viscosus* and *M. cremoris-viscosi*).[2]

Most of them produce brilliantly colored colonies but the function of their pigment is not well understood. Pigment formation is often a rather variable characteristic.

Occasionally one or another species of *Micrococcus* is found apparently causing a pathological condition but may, in such a case, be regarded as what Theobald Smith called an "opportunist"; *i.e.*, an organism (of any sort) which is able to take advantage of some special or unusual combination of circumstances (such as the weakened condition of a patient or an old ulcer) favorable to its development.

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* Gaffky was a famous bacteriologist who first described these organisms.

† Sarcina is from a Latin word meaning packet.

PATHOGENIC MICROCOCCI[3]

Because the name *Staphylococcus* is one of long standing and common usage, it will be continued here in a nonspecific sense to mean pathogenic micrococci. Staphylococci have the general properties of other micrococci. In general, the individual cells of staphylococci are smaller than those of the other Micrococcaceae (Fig. 252). Pathogenic staphylococci generally: (1) ferment mannite, (2) liquefy gelatin and (3) produce coagulase, an enzyme which causes oxalated blood plasma to clot. However, these properties are neither constant nor exclusive among staphylococci, and it is sometimes very difficult to make a distinction between staphylococci and micrococci. True staphylococci, especially *S. aureus,* often produce very hemolytic colonies when cultivated on the surface of blood agar. However, many non-pathogenic species are also very hemolytic.

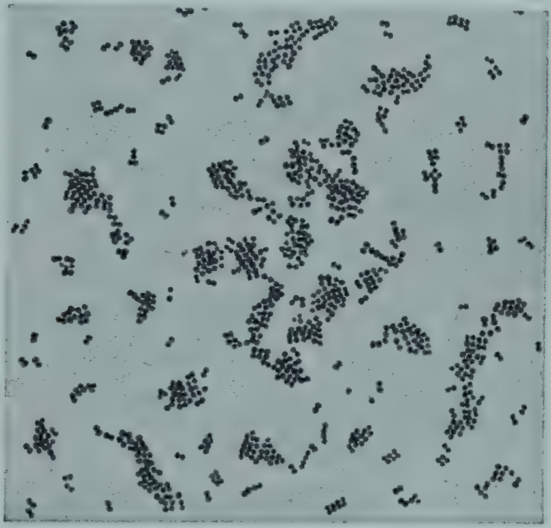

Fig. 252. *Staphylococcus aureus* from 24-hour culture on agar, stained with safranin (× 900). (Ford.)

Verwey has shown that strains of micrococci may be roughly differentiated by means of the electrophoresis apparatus. Pathogenic strains generally move slowly, the non-pathogenic strains rapidly. The pathogenic strains are especially resistant to 7.5 per cent NaCl in media.[4]

The true staphylococci are regarded as parasites and are usually to be found on the skin or mucous membranes of the animal body, especially of the nose and mouth, where they often occur in large numbers even under "normal" conditions. Two principal species may be mentioned: *M. aureus,* distinguished by its golden-yellow pigment and notorious as the cause of mastitis of cows, and of boils, carbuncles, and internal abscesses in man; and *M. albus,* recognized by its paper-white pigment, opaque colonies, and universal presence on the human skin. The golden-yellow species are much more dangerous than the white species, although the latter often cause infections of the skin and occasionally severe abscesses.

The more pathogenic staphylococci, especially *M. aureus,* produce a variety of pathological effects. There are various names for the toxin (or toxins) on which these effects depend. It is not entirely clear just how many separate toxins there may be. The effects have been variously described as due to (1) *lethal toxin* (kills animals, for example, rabbits, very quickly, [a few minutes] after intravenous injection); (2) two sorts of *hemotoxin* (alpha hemotoxin, which hemolyzes rabbit *and* sheep erythrocytes and beta hemotoxin, which does not hemolyze rabbit cells); (3) *dermotoxin* (produces a necrotic area in the skin when injected intradermally); (4) *coagulase* (brings about coagulation of citrated blood); (5) *fibrinolysin* (digests fibrin); (6) *leukocidin* (kills leukocytes); (7) *enterotoxin* (produces acute gastroenteritis when ingested by human beings and some of the lower animals).[5]

Most of these activities of staphylococcus cultures or culture filtrates are easily demonstrated. Animal injections serve for the demonstration of the lethal and dermonecrotic factors, and hemolysis and leukocyticidal activities are readily demonstrated by mixing suspensions of the appropriate cells in test tubes with the tested culture and observing the effect microscopically. The fibrinolysin test is similar to that used for streptococci. The test for coagulase is a simple one, and valuable because coagulase production seems to be most closely related to virulence or pathogenicity of the cocci.[6] All that is necessary is to mix, in a small tube, a loopful of young growth from an agar culture of the tested organism and 0.5 ml. of freshly prepared citrated or oxalated human plasma diluted about 1:3 with saline or broth. Coagulation will occur in an hour or so at 37° C. It sometimes progresses slowly. It seems that different human bloods differ in coagulability by coagulase.[7]

There is some evidence that the lethal, dermonecrotic and alpha hemotoxic effects are due to the same factor. However, there is conflicting evidence on this point, so that definite statements cannot be made. Coagulase and fibrinolysin seem entirely independent substances, and leukocidin may be. The enterotoxin is a separate and distinct fraction of the toxic principle of staphylococci. It is thermostable; the others thermolabile.

PATHOGENIC GAFFKYA

An interesting instance of pathogenicity of this organism, occasionally causing minor infections in man, is seen in a disease of lobsters caused by *Gaffkya*. The disease has a high fatality rate. The diseased lobsters have a pink discoloration on the ventral side of the abdomen; the blood, normally blue, is pink and watery. The organisms are found in large numbers in the blood and tissues and Koch's postulates have been demonstrated with them. The tetrads in the lesions are characteristically heavily encapsulated.[8]

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Chapter 32

SPHERICAL BACTERIA. II. THE STREPTOCOCCEAE

Three genera are included in this tribe: *Streptococcus, Diplococcus* and *Leuconostoc*. This chapter will deal in detail with the first.

As the name implies, streptococci grow in chains (Fig. 253). Sometimes chains of only two, three to 30 or 40 cells occur; in other species very long chains of several thousand cocci may be observed. Some interesting details of how streptococci hang together in chains have been revealed with the electron microscope (Fig. 254). *Chain formation can be most accurately observed only by cultivating the streptococci in pure culture in broth and observing them in stained smear or hanging-drop preparations.* All are gram-positive (although some of them appear to be gram-positive only when cultivated in blood or serum medium), and many of them are pathogenic. All grow on infusion agar or in infusion broth, but some of them, especially the pathogens, grow better if blood or serum be added. Some are quite proteolytic, especially certain of the pathogenic type which actively digest dead tissues and fibrin, and harmless streptococci of the vagina and intestine which liquefy gelatin. All streptococci ferment dextrose without formation of gas except the genus *Leuconostoc* which produces CO_2.

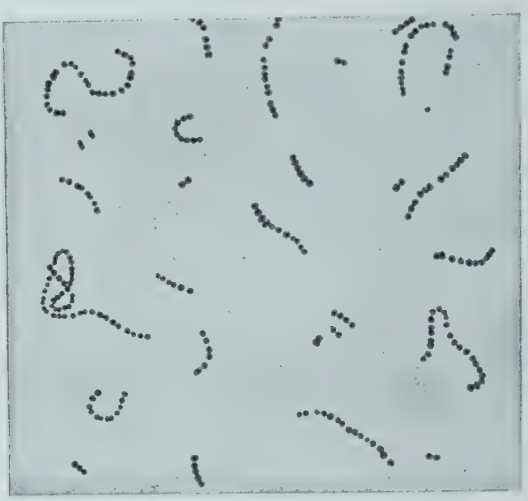

Fig. 253. Typical streptococci (× 900). (Ford.)

Interrelations of Streptococceae. The first two genera of this tribe (*Diplococcus* and *Streptococcus*) are closely related in morphology, chain formation, capsule production, dependence for growth upon blood or serum media or other complex organic nutrients, and pathogenic properties. Representative species of each genus are found in the normal mouth and upper respiratory tract. It seems of doubtful utility to classify them in separate genera. However, members of the genus *Diplococcus* are differentiated from most streptococci by the conical shape of the cells, by growth in pairs and chains of pairs, as well as several physiological peculiarities: solubility in bile, ability to ferment inulin, production of *lobar* pneumonia in human beings, and possession of large and easily stained capsules. They are discussed more fully in a later chapter.

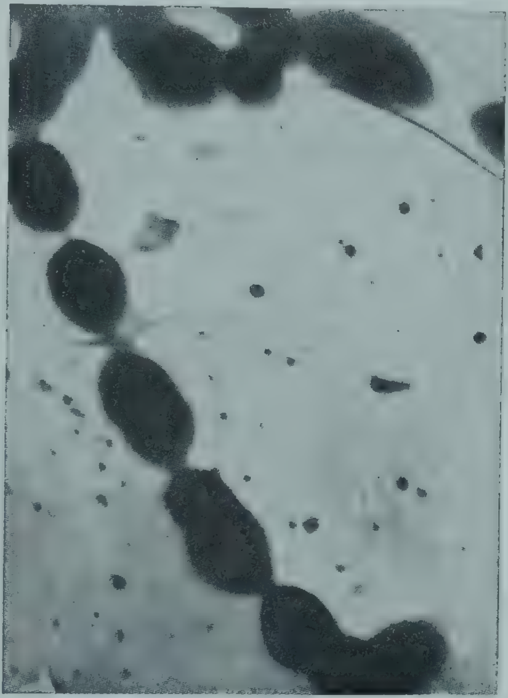

Fig. 254. Electron microscope picture of streptococci (× 20,000). (Zworykin.) Note the thin protoplasmic filaments connecting the cells. Compare with Figure 253.

Some species of *Streptococcus* are highly pathogenic and never live free in nature. There are also many streptococci of little or no pathological significance, but of great importance in the dairy industry, which are found in the intestines of man and animals, and in milk, cheese, etc. All streptococci are thus seen as rather closely related to the animal body.

The members of the third genus of chain-formers (*Leuconostoc*) are saprophytic and are found in situations unrelated to a parasitic existence or pathogenic activity, like vats in sugar refineries and in fermenting sauerkraut. They are widely distributed in nature on plants, soil, etc.

Classification of the Genus Streptococcus. One of the most difficult problems in bacteriology is the systematic subdivision of a family or tribe into genera and species, and the difficulty of the problem is well illustrated by the streptococceae. Division of the tribe into the three genera (*Streptococcus, Diplococcus* and *Leuconostoc*) is not excessively difficult, as noted in the preceding paragraphs. Neither does division of the genera *Diplococcus* and *Leuconostoc* into species present very serious problems.*

TABLE X

CLASSIFICATION OF THE STREPTOCOCCEAE

Cocci forming definite chains

- Homofermentative* (Lactic acid formed; no CO$_2$)
  - Genus *Streptococcus*
    - Not lysed by surface tension reducents
      - *Lactic group* (Dairy products)
        - No pathogens
        - Growth at 10° C. No growth at 45° C.
      - *Enterococcus group* (Intestinal tract)
        - Few, if any pathogens
        - Growth at 10° C. Growth at 45° C.
      - *Viridans group* (alpha type of hemolysis)
        - Many pathogens
        - No growth at 10° C. Growth at 45° C.
      - *Pyogenic group* (beta type of hemolysis)
        - Many pathogens
        - No growth at 10° C. No growth at 45° C.
  - Genus *Diplococcus*
    - Lysed by surface tension reducents, including bile
      - Moderately large capsules; conical cells; ferment inulin; cause lobar pneumonia
- Heterofermentative† (CO$_2$ formed)
  - Genus *Leuconostoc*
    - Heavily encapsulated; saprophytic

* *Homofermentative:* Carbohydrate fermentation in which a single acid product of the fermentation is formed; in this case lactic acid. Minute quantities of other acids, alcohols, etc., have been said to occur in these fermentations but lactic acid constitutes a high percentage of the mixture.

† *Heterofermentative:* Differentiated from homofermentative by the formation of a mixture of acidic and volatile compounds during the fermentation of carbohydrates.

Subdivision of the genus *Streptococcus*, however, presents some of the major problems of bacteriology. A primary subdivision is made into 4 large groups, as shown in Table X (Lactic, Enterococcus, Viridans and Pyogenic), on the basis of certain physiological properties. Subdivision of these groups depends on certain biochemical and immunological reactions as follows.

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* Perhaps we here underappreciate the anguish of the taxonomist.

Blood-agar Types. A very useful method for primary differentiation of streptococci (genus *Streptococcus*) is cultivation in blood-agar plates.[1] The differential criterion is the effect of the colonies of streptococci on the red blood cells in the agar.

In order to determine this, a tube containing about 15 ml. of melted infusion agar, cooled to about 45° C. (still fluid), is inoculated with a loopful of pus, milk, broth culture or other material containing the desired streptococci, and about 5 per cent sterile blood is added and well mixed with the agar. The mixture is poured (Fig. 255) into plates and incubated at 37° C. for twenty-four hours. Two main types of streptococci are recognized, the alpha and beta hemolytic types.

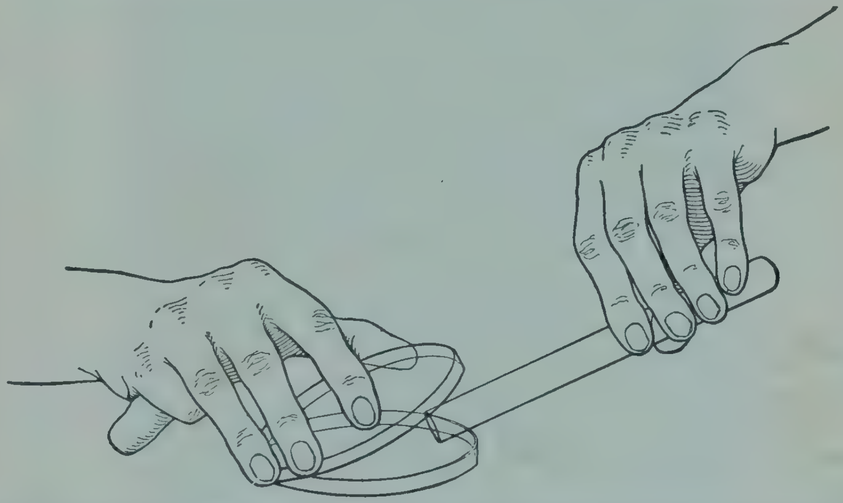

Fig. 255. Method of pouring a plate. The cotton plug can be held as shown, or can be grasped between the ring and little finger.

Alpha Type. Colonies of alpha-type hemolytic streptococci in blood-agar pour-plates prepared as described above, are seen to be surrounded by a zone of hemolysis and also by a zone of discolored erythrocytes close in around the deep colonies, the latter clearly *visible only with the low power of the microscope*. These erythrocytes have a green or brownish-green color. Beyond this inner ring of discolored cells the outer zone of clear hemolysis may be of great or small extent, and may sometimes be so small as to be invisible, but usually widens on refrigeration of the plate.

When viewed with the naked eye, alpha-type colonies having a *wide* zone of hemolysis are sometimes mistaken for colonies of hemolytic streptococci of the beta type, especially if the inner, green zone be very narrow, as often happens. *Only the use of a microscope can be relied upon to make the distinction and only colonies which are deep in the agar* are always thus characterized, surface colonies sometimes producing deceptive appearances. The green-producing varieties of hemolytic streptococci (*hemolytic streptococci of the alpha type*) are often loosely called *Str. viridans* (Fig. 256). Sometimes, upon alternate refrigeration and incubation, several concentric zones of hemolysis and greenish discoloration may be produced. These must not be confused with the double-zone beta type streptococci described below.

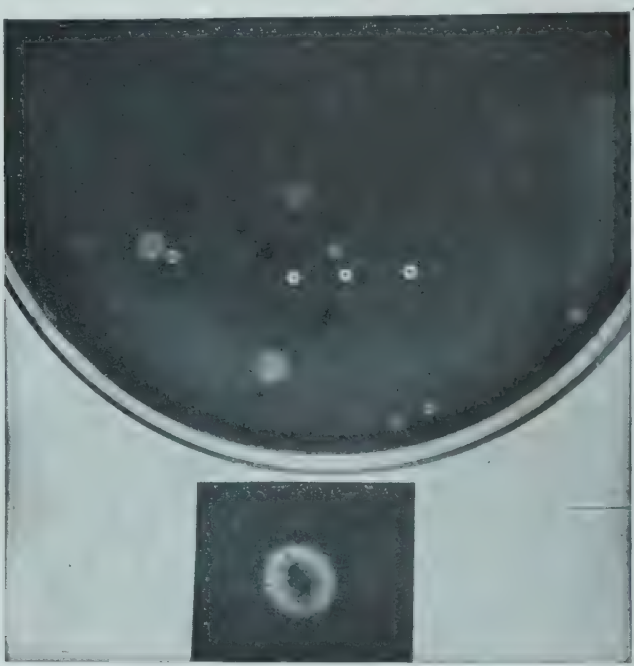

Fig. 256. Natural size photograph of blood agar plate showing colonies of *alpha-type hemolytic streptococci* (*Streptococcus viridans*). Each colony is surrounded by a small clear zone. In the center, close around the colony, is a zone of greenish or brownish corpuscles. Sometimes this zone is so wide as to mask the clear part. Often it is so narrow as to be visible only with the microscope as shown in the small insert. The colony may then be mistaken for a true beta type *Streptococcus* colony. (Courtesy of Dr. J. Howard Brown, Johns Hopkins University.)

Beta-type Hemolytic Streptococci. The hemolytic zones of streptococci of this type in blood-agar plates are seen to be entirely clear and free from any intact erythrocytes. Such streptococci are loosely spoken of under the general term of "streptococcus hemolyticus or "hemolytic strep." They may more accurately be termed *hemolytic streptococci of the beta type* (Fig. 257).

Double-zone Beta-type Streptococci. A very interesting subdivision of the beta-type streptococci is seen in the double-beta-zone-producing subtype described by Brown in 1937.[2] Certain species, almost exclusively of bovine origin, after producing a zone of hemolysis like that of other beta-type streptococci, on standing at room temperature or on refrigeration produce a second ring of hemolysis separated from the first by a ring of red erythrocytes (Fig. 258). All double-zone beta-type hemolytic streptococci so far described have belonged to Lancefield's group B (see page 551) but not all group B strains are known to produce double zones.

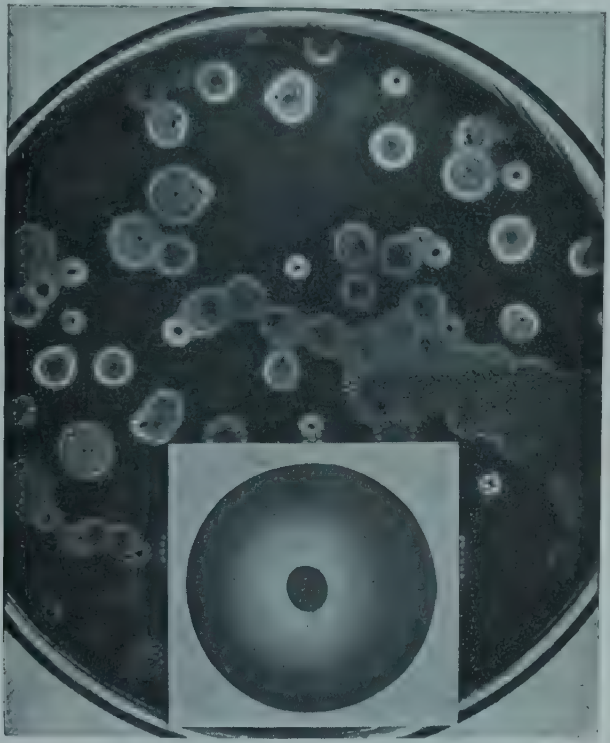

Fig. 257. Natural size photograph of blood agar plate showing colonies of beta-type hemolytic streptococci. Each colony is surrounded by a perfectly clear zone in which all the blood corpuscles have been completely destroyed. The insert shows one of the colonies much enlarged. (Courtesy of Dr. J. Howard Brown, Johns Hopkins University.)

Gamma Type. When colonies of streptococci in blood-agar plates show no visible change in the blood cells surrounding the colony they are said to be of the gamma or indifferent or non-hemolytic type.

With respect to the groups listed in Table X, all three blood-agar types are found in the lactic and enterococcus groups. The viridans group comprises alpha type streptococci and the pyogenic group is made up of beta type streptococci.

Other Subdivisions of Streptococci. Each of the types of streptococci distinguished, as described above, according to its action on blood agar may be again divided, on the basis of still other properties, into what are variously termed divisions, varieties or species, or subspecies, depending on one's conception of the meaning and scope of these terms. Specific names are given to some of the better known varieties of each division, on the basis of source or other properties. For example, when beta-type hemolytic streptococci were first discovered, it was customary to name them according to the disease they were thought to cause. *Str. pyogenes* (pyogenes = pus-producing) was a name early given to streptococci causing suppurative infections of various parts of the body. This name is accurately descriptive and still holds and *Str. pyogenes* is the type species of the genus. However, it has since been found that the beta-type hemolytic streptococci causing one disease can often cause other diseases interchangeably with other beta-type hemolytic streptococci. The tendency at present is, therefore, to refer simply to *hemolytic streptococci of the beta*

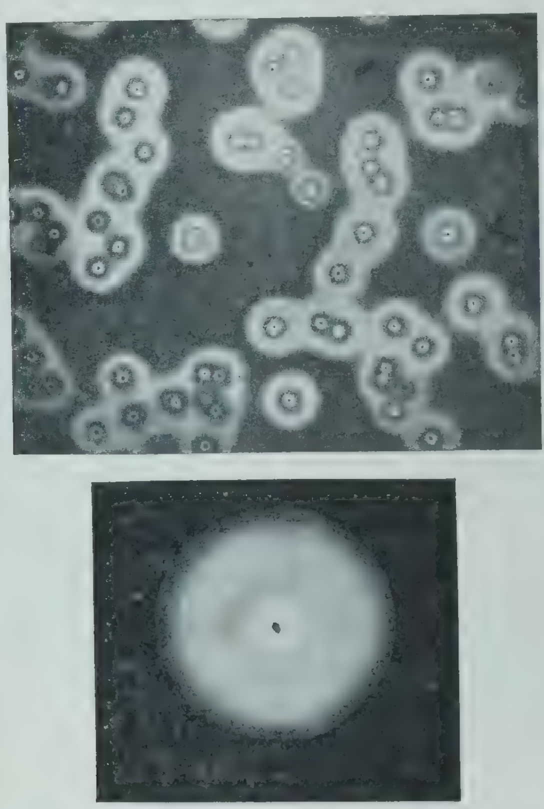

Fig. 258. Upper, double-zone beta-type hemolytic streptococcus colonies in blood agar, incubated 24 hours at 37° C. and then refrigerated overnight. Lower, single colony enlarged 8 times. (Brown, J. Bact., vol. 34.)

type, stating the source or, better, the Lancefield group or type (see page 549).

Fermentative Varieties. Attempts to establish species of streptococci on the basis of fermentation reactions have led to great confusion. Many of the "species" established by this method are invalid because various fermentative types occur in any of the groups established by other means. Only a few specific fermentation tests are of use as applied to some of the finer distinctions among varieties of these organisms established on other grounds.

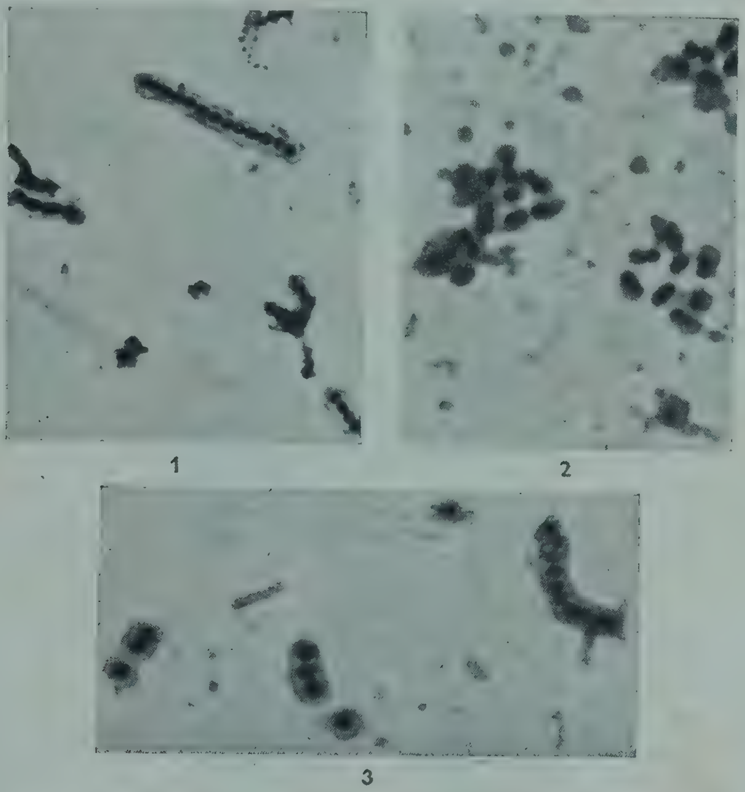

Fig. 259. Streptococci of the beta type stained by Hiss's method for capsules. *1,* Group A; *2,* group B; *3,* group C. (After Hobby and Dawson.)

SEROLOGICAL GROUPING OF HEMOLYTIC STREPTOCOCCI

So far as the beta-type hemolytic streptococci are concerned, serological grouping has proved most informative.

Lancefield Groups. The most useful means of studying beta-type hemolytic streptococci have been developed by Lancefield.[3] She made extracts of massive cultures of different strains of these bacteria by means of hot N/20 HCl. These extracts contain specific, somatic, antigen substances. The clear extracts are used as antigens in precipitin tests. The precipitin-containing serum used in these tests is prepared by injecting rabbits with the sedimented cocci from selected, 18-hour-old broth cultures treated with 0.2 per cent formaldehyde.

By making tests[1] with antisera and extracts of beta-type hemolytic streptococci from various sources, it was found that there were fairly distinct precipitin *groups* of these streptococci with respect to origin. Lancefield designated these groups by letters of the alphabet: A, B, C, D, E, F, G, etc., according to source or other characters.

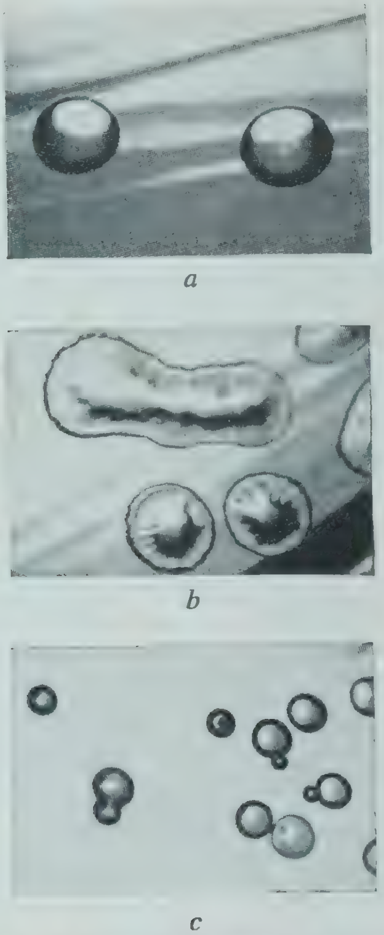

Fig. 260. Colony variants: *a*, mucoid; *b*, matte; *c*, glossy. (Lancefield, Rebecca C., in The Harvey Lectures, Series 36, 1940–41.)

Group A Streptococci. All strains of group A yield a carbohydrate (in the extraction process described) which acts as the antigen in the precipitin test. It is called a *C* (Carbohydrate) *substance*. A single kind of C substance is common to all members of group A.

Biological properties distinguishing group A streptococci from all other groups are shown in Table XI. Special attention is called to their reactions on the substances trehalose and sorbitol, the former of which is fermented, the latter not. The special value of this fact will be pointed out later (see page 552).

Group A streptococci include the strains causing scarlet fever, septic sore throat, empyema, puerperal sepsis, and many other serious diseases in human beings.

Types and Colony Variants. Two colony forms of beta type hemolytic streptococci of *group A* have been discerned: a smooth, regular, *glossy* form and a dull, slightly irregular form called *matte* or M.

TABLE XI

SUBDIVISIONS OF THE HEMOLYTIC STREPTOCOCCI

Action on Blood Agar

Alpha type	*Beta type*
("Viridans" group)	*Lancefield groups* (C substances):
Also a few enterococci and lactic streptococci	A. Dangerous pathogens (Human) (Fibrinolytic) (*Str. pyogenes,* etc.) *Glossy*—T substance (agglutinins—type specific) (± 40 types) *Matte*—T substance (agglutinins—type specific) M substance (agglutinins, precipitins, opsonins, protective antibodies, etc., type specific) (± 40 types)
	B. Mainly bovine pathogens—mastitis (*S. mastitidis*) *S substance*—precipitin types *Double zone*—beta type colonies *Hippurate hydrolysed*
	C. Human pathogens, Tre.+, Sorb.–, Glyc.+ (Fibrinolytic) Animal pathogens, Tre.–, Sorb.+, Glyc.–^{4a} (*S. equi*)
	D. Enterococci (*S. zymogenes*)
	E. Dairy products
	F. Human throat. Produce minute colonies
	G. Human pathogens (Fibrinolytic)

Glossy variants are usually of low virulence; matte of higher virulence. A mucoid type has also been differentiated (Fig. 260). Only the matte and glossy colonies are discussed here.

T substances. Both of these variants contain a protein antigen called T (for type) substance which engenders *only agglutinins* when injected into rabbits. Now not all strains of group A hemolytic streptococci contain the same *kind* of T substance—in fact there are some 40 or more *different* kinds or types and 40 or more corresponding type-specific agglutinins.*

---

* These are probably responsible for Griffith's observation of various agglutinative "types" of hemolytic streptococci, most of them in Lancefield group A.

M substances. The *matte* variants of each of these 40 or more types contain, in addition to T substance, proteins which engender not only agglutinins, but also precipitins and protective antibodies. They are called M (for matte) substances. *These M substances have the same type specificity as the T substances.** Type-specific precipitin tests

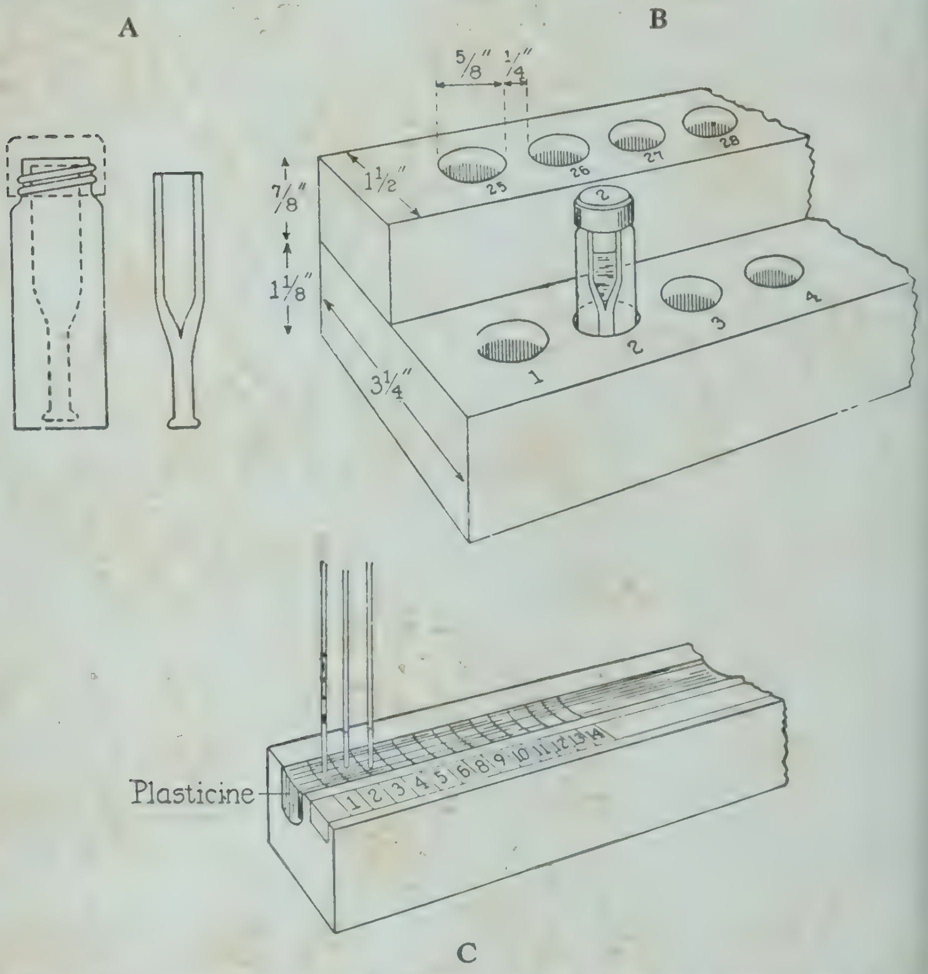

Fig. 261. *A*, Serum container in screw cap vial; detail to show shape. *B*, One serum container in place. *C*, Capillary pipette stand with three pipettes in place precipitate in first tube (serum type 1). (Swift, H. F., Wilson, A. T., and Lancefield, R. C., in J. Exp. Medicine, vol. 78.)

may, therefore, be done by using appropriate antisera† and M-substance-containing extracts from the matte streptococci. These extracts are prepared as described for grouping (A, B, C, etc.). The relationships of streptococcus groups and types are shown in Table XI.

* Usually.

† The preparation of these requires great skill and experience.

The M precipitin test has two great advantages over the T agglutination test in that (1) many strains persist in granular growth which interferes with agglutination tests; (2) some strains of different M types contain identical T substances. By doing precipitin tests with the M substance, both of these difficulties are avoided.

Because of the difficulty and expense of preparing M type sera, the tests are not run in the usual small test tubes which require perhaps 0.2 ml. of serum for a test, but in *capillary* tubes, which require only about 0.01 ml. of serum per test[5] (Fig. 261).

Significance of Streptococcus Typing. Evidence has accumulated that, in epidemics of streptococcal disease such as scarlet fever and septic sore throat, single types of streptococci tend to predominate and that resistance to streptococcal infection is type specific. For example, one may have resistance to a type-6 (M type) streptococcus epidemic yet succumb in a type-19 epidemic with the same disease. The importance of typing in the study of methods by which streptococcal disease is spread can scarcely be overestimated.[6, 7, 8] The whole problem of the control of rheumatic heart disease appears bound up with the epidemiology of streptococcal infection.[9] The therapeutic value of streptococcus typing is evident in the fact that some types are resistant to sulfonamide drugs; further studies are required relative to penicillin, etc.[10]

Isolation of Hemolytic Streptococci. Isolation of streptococci, pneumococci and many other organisms of the respiratory tract, ulcers, wounds, abscesses, etc., is often done simply by rubbing over the surface of a blood agar plate a swab which has been passed gently but firmly over the patient's tonsils, nasopharynx, abscess or other lesion. After twenty-four to forty-eight hours of incubation at 37° C. colonies distinctive of the organism sought are looked for. This requires knowledge and experience and is not always satisfactory. The blood-agar pour plate previously described is the best procedure. The method of Pike has also given good results. Prepare beef heart infusion broth containing tryptose, 1 per cent; dextrose, 0.02 per cent; rabbit blood, 5 per cent. This is placed in 2 ml. amounts in small tubes. Just before use add to each tube: NaN_3, 1:1000, autoclaved, 0.15 ml.; crystal violet, 1:25,000, autoclaved, 0.1 ml. Rub the swab on the patient's lesion and place it in a tube of broth. Incubate at 37° C. for eighteen to twenty-four hours. Then streak a blood-agar plate with the broth. The NaN_3 inhibits gram-negative contaminants, the crystal violet inhibits many staphylococci and other unwanted organisms. Dextrose favors the growth of the streptococci.[11]

Group B Streptococci. The organisms of this group resemble those of Group A in possessing in common a group-specific "C" substance which is antigenically different from that of group A cocci. Group B cocci also are separable into "types" by means of a second antigenic substance analogous to the "M" substance of group A.

The group B streptococci differ from those of all other Lancefield groups in hydrolyzing sodium hippurate. The very striking property of double-hemolytic-zone production by group B streptococci has been referred to above (page 545).

Hemolytic streptococci of group B are usually of bovine origin and harmless for man, but are occasionally found in human infections. *Str. agalactiae,* an important member of group B, is of particular interest to the farmer because it is one of the common causes of mastitis in cows and because it is sometimes confused with dangerous group A streptococci which may also cause mastitis in cows and, when discharged in large numbers in the milk, may produce widespread outbreaks of scarlet fever and septic sore throat.[12, 13, 13a] The group A streptococci gain entrance to the udder from the hands of milkers who have the organisms in their throats. When the infection of the udder due to either group of cocci is acute, the streptococci may be found in the milk by inoculating melted infusion agar with diluted milk and pouring it, mixed with blood, into Petri plates as already described. The hemolytic colonies appear after twenty-four hours' incubation at 37° C.

Differentiation of Group A and Group B Streptococci. The bacteriologist, investigating the cause of an epidemic, is often faced with the problem of determining whether hemolytic streptococci in milk are of the usually harmless bovine group (B) or of the dangerous, human group (A). Sure differentiation is most quickly and easily done by the Lancefield *group* precipitin test, and the observation of double-zone beta type hemolytic colonies. Data collected by Evans indicate that most milk-borne outbreaks of streptococcal disease in human beings are caused by Group A organisms of types *1, 11, 12,* and *27.* These types appear to have a special ability to invade the bovine udder.[14]

Group C Streptococci. This group is of a dual nature with respect to origin, containing (1) strains of human origin culturally indistinguishable from group A cocci. They are not such dangerous pathogens. (2) Strains from various animal sources. The latter rarely occur in human infections and may be divided into two subgroups on the basis of fermentation reactions. One, represented by the species called *Str. equi* because it is found especially in diseases of horses, ferments neither sorbitol nor trehalose. The second, occurring in diseases of horses as well as of animals such as cattle, guinea pigs, rabbits and ferrets, ferments sorbitol but not trehalose. The group C strains of human origin ferment trehalose but not sorbitol. They also produce human fibrinolysin, which the animal strains do not (see

TABLE XII

SOME DIFFERENTIAL CHARACTERS OF STREPTOCOCCI

Group Designation	Action on Blood-agar Plate	Growth at		Survival of 60° C.	Growth in			Representative or Important Species or Varieties	Lancefield Group	Ferments					Acid in Dextrose Broth	Attacks		Human Fibrinolysin	Inulin Fermented	Bile-soluble
		10° C.	45° C.		NaCl 6.5%	pH 9.6	0.1% meth. blue.			Lact.	Man.	Sal.	Tre.	Sorb.		Sod. Hip	Gel.			
Beta-type hemolytic streptococci or "*Str. hemolyticus*" (pyogenic group)	β	−	−	−	−	−	−	Str. pyogenes	A, E F, G	+	−	+	+	−	little	−	−	+	−	−
								Str. agalactiae	B	+	−	+	+	−	much	+	−	−	−	−
								Str. equi	C	−	−	+	−	−	little	−	−	−	−	−
								"human C"		+	−	±	+	−	mod.	−	−	+	−	−
								"animal C"		+	−	+	−	+	little	−	−	−	−	−
Alpha-type hemolytic streptococci "*Str. viridans*"	α (some γ)	−	+	− (some +)	−	−	−	Str. salivarius		+	−	+			much	−	−	−	−	−
								Str. thermophilus*		+	−	+			much	−	−	−	−	−
								Diplococcus pneumoniae†		+	−	−			much	−	−	−	+	+
Gamma or indifferent type streptococci or "lactic streptococci"	γ	+	−	+	−	−	+	Str. lactis‡		+	±	±			much	−	−	−	−	−
								Str. cremoris‡		+	−	±			much	−	−	−	−	−
"Enterococci"	α	+	+	+	+	+	+	Str. faecalis		+	+	+	+	+		−	−	−	−	−
	β							Str. liquefaciens		+	+	+	+	+		−	+	−	−	−
	γ							Str. zymogenes	D¶	+	+	+	+	+		−	+	−	−	−

* Distinguished especially by (*a*) growth at 50° C. and (*b*) failure to ferment maltose, from all other streptococci in this list. Is a great nuisance because resistant to pasteurization.

† Does not grow at 45° C. and always produces alpha type colonies (except anaerobically).

‡ These are differentiated by the ability of *Str. lactis* to grow at 40° C. and in media with a salt concentration of 4 per cent or a *p*H of 9.2. *Str. cremoris* cannot grow under these conditions.

¶ Beta hemolysis in blood agar.

Tables XI and XII). Fibrinolysin is an enzyme which digests human blood clot. This is of importance in streptococcal infection (see pages 357 and 739).

Group D Streptococci. These streptococci, like those of group C, possess a common antigen among themselves that is probably not a carbohydrate. They are a well-differentiated sort of streptococci, being really members of the group of enterococci, which are discussed elsewhere in this chapter. They differ markedly from cocci of groups A, B and C in a number of important biochemical and physiological properties which are shown in Table XII.

Group G Streptococci. These strains are possibly related antigenically to some group C strains, and closely resemble group A strains in important biochemical properties. They are generally not highly pathogenic, but vary in this respect. They are frequent in human infections. They produce fibrinolysin (see Table XII).

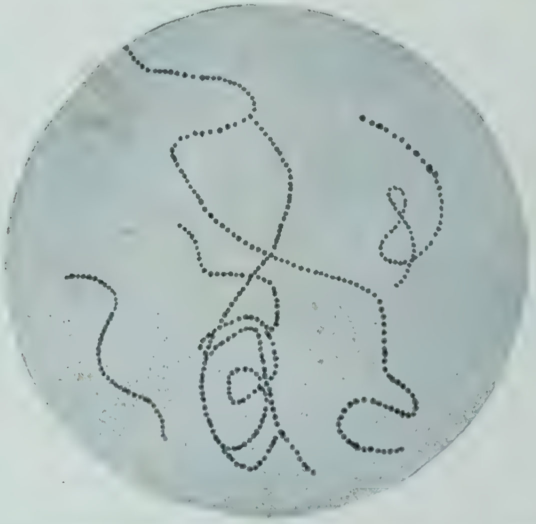

Fig. 262. Hemolytic streptococci of the alpha type (*Str. salivarius*), cultivated in broth from an abscessed tooth. Very long chains (× 900).

Groups E, F, H and K. These groups contain such small numbers of strains that they will not be discussed in detail. They do not appear to have any special pathological significance, but have been found occasionally in animals and human beings, and also in dairy products.

Alpha and Gamma Types. The alpha-type hemolytic (or "viridans") streptococci and the gamma or nonhemolytic varieties may be considered under three subheadings: the viridans streptococci proper; the lactic group, common in dairy products; and the so-called enterococci.

denizens of the intestinal tract. The subdivisions are not so clearly differentiated as might appear; that is, some of the alpha type species appear to form both alpha and gamma colonies, and some gamma species are found in the so-called viridans group as well as in the lactic group and among the enterococci. For this reason alpha and gamma types from all sources are included in one discussion.

"Viridans" Streptococci. This group includes mainly the common streptococci of the alimentary tract. These are represented by *Str. salivarius* of the human mouth (Fig. 262). The colonies of this species in blood agar are definitely of the alpha type. The effects of *Str. salivarius* on lactose, mannite and salicin are like those of *Str. pyogenes* which shows how little differential value is to be placed on fermentative properties of this sort. *Str. salivarius* might be called the "type species" of the "viridans group." Similar varieties are found in the intestinal tract of the horse (*Str. equinus*) and cow (*Str. bovis*). A related species growing well at high temperatures (65° C.) occurs in milk (*Str. thermophilus*). It gives much trouble to commercial milk pasteurizers because, since it is not destroyed by pasteurization and grows to large numbers in pasteurizing vats, it appears in the milk upon examination by the health department and casts doubt on the efficacy of the pasteurization process. It is not infectious.

Morphology of Alpha Type Streptococci. Often the alpha and gamma types of hemolytic streptococci show a considerable degree of pleomorphism. They tend particularly to become elongated and often produce involution forms resembling misshapen bacilli; so much so that they have frequently been mistaken for diphtheria bacilli. Some workers have seen a relationship between these streptococci and the corynebacteria. That this view is not sound has been pointed out by Lamanna.[15]

Lactic Streptococci. This group contains *Str. lactis,* the common milk-souring streptococcus, useful in cheese-making, and of which there are several varieties (or species, depending on one's definition of a species). These are usually of the gamma type in blood agar but may produce green colonies. *Str. lactis* is always present in market milk, even of the best quality. It occurs in cow dung and its entrance into the milk is easily explained. It is quite harmless to man.

It occurs chiefly in short chains or pairs and the cells tend toward an oval shape. It grows rapidly in the milk at summer temperatures, usually overgrowing and suppressing the development of other organisms, some of which might otherwise cause the milk to putrefy. It ferments the lactose, producing lactic acid and the not unpleasant flavor characteristic of "cottage cheese." "Cottage cheese" is simply the casein of milk coagulated and flavored by the lactic acid and certain other aromatic products of the fermentation of lactose, such as diacetyl. *Str. lactis* has no ability to hydrolyze proteins like casein or gelatin.

STREPTOCOCCI RELATED TO STREPTOCOCCUS LACTIS. There are numerous closely related streptococci, some of them forming gas during the fermentation of lactose, and all of them forming lactic acid. One of these is *Str. cremoris.* Pure cultures of this organism called "starters," are used in making butter and cheese, the object being to ensure that the milk or cream is soured by an organism which yields a pleasant flavor. *Str. lactis* may be termed the "type species" of the "lactic group." The differentiation of gas forming lactic streptococci from *Leuconostoc* appears difficult. The two groups appear to overlap considerably. True *S. cremoris* does not form CO_2.

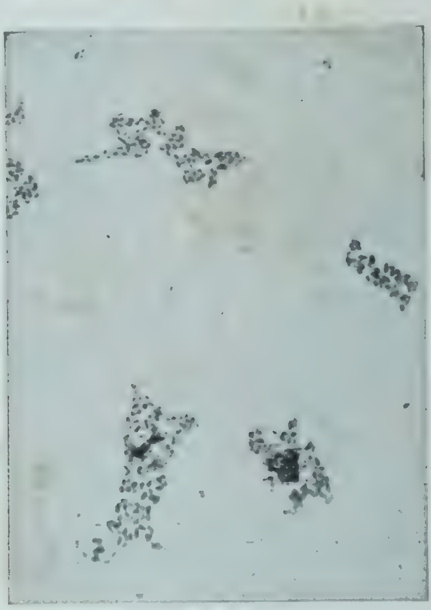

Fig. 263. *Streptococcus liquefaciens* (× 900). (Frobisher and Denny, J. Bact., vol. 16, 1928.)

The "Enterococci." This subdivision of the genus *Streptococcus* comprises a number of species with wide tolerance of heat and cold and other influences unfavorable to other streptococci such as 6.5 per cent NaCl, bile, *p*H 9.6,[16] and conditions of life in feces. The enterococci are commonly found in the intestinal tract and are represented by *Str. faecalis.* Colonies of this organism usually produce alpha type zones of hemolysis in blood-agar plates. Other species of enterococci produce *beta* type colonies, and some closely related species produce no change in blood agar (gamma type). The group is seen, therefore, to be somewhat heterologous (see Table XII).

Related to *Streptococcus faecalis* are *Str. liquefaciens* and *Str.*

zymogenes, the last differing from *S. liquefaciens* only in producing beta type zones in blood agar. *Str. zymogenes* possesses an antigen which relates it to the beta hemolytic streptococci in Lancefield's group D (see page 553).

The enterococci are closely related, both in properties and habitat, to the lactic group, but they can be differentiated by temperature relationships (see Table XII). The lactic group are not proteolytic, while *Str. liquefaciens* and *Str. zymogenes* hydrolyze proteins. They also give rise to aromatic and bitter flavors in cheese. They occur in cow dung and have been found in sewage and in certain pathological conditions.[17] All of the enterococci have a tendency to produce short chains and pairs of plump, ovoid cocci, and are commonly found in clumps suggestive of staphylococci (see Fig. 263).

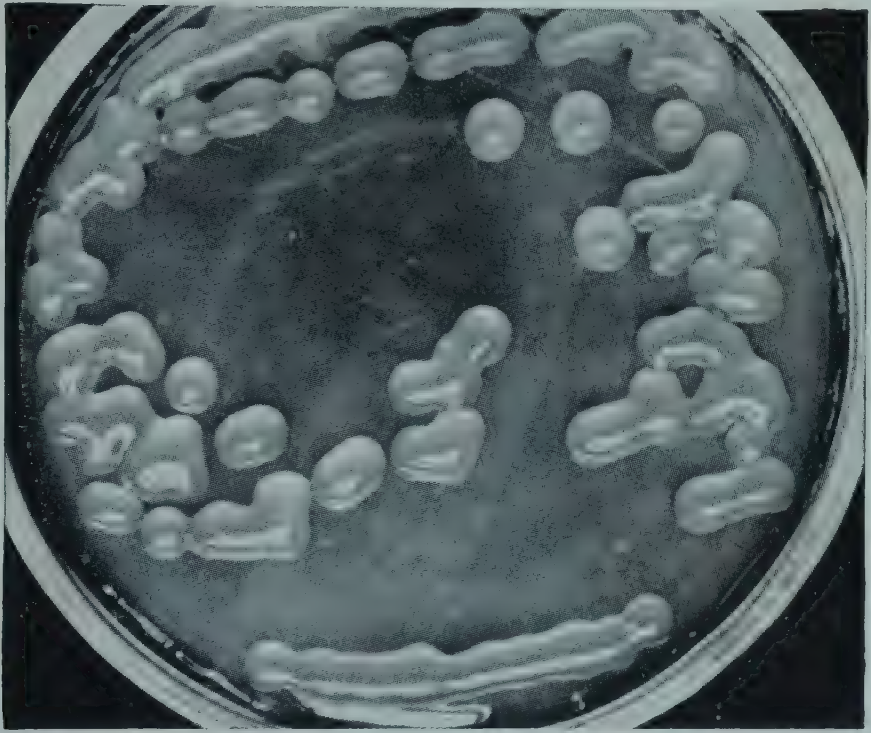

Fig. 264. *Leuconostoc mesenteroides.* Strain 200, D type. Three-day culture on 10 per cent raw sugar agar, incubated at room temperature. (McCleskey, C. S., Faville, L. W., and Barnett, R. O., in J. Bact., vol. 54.)

Genus Leuconostoc. These organisms produce spherical cells in short chains which secrete thick masses of gummy polysaccharide about themselves (Fig. 264). They are about 2 microns in diameter. *Leuconostoc* differs from other streptococci in forming carbon dioxide from dextrose.

A detailed study of biochemical and immunological properties of

L. mesenteroides, a common species, has shown that there are various types, differentiated serologically and by the amount of gum formed, amount of CO_2 produced, etc. Varieties producing no CO_2 would resemble the lactic streptococci. However, all *Leuconostoc* are distinctly heterofermentative, grow in the presence of 4 per cent NaCl, and showed a preference for higher temperatures for growth than do lactic streptococci.[18]

Polysaccharide Synthesis. Most of the Streptococceae can form carbohydrates (polysaccharides) from glucose, sucrose, etc., often as a slimy, capsule-like outer covering. As already shown, these carbohydrates often confer immunological specificity on the organisms producing them and form the basis for some of our most useful diagnostic tests (precipitin tests).

Polysaccharide synthesis is a particularly prominent feature of *Leuconostoc.* These organisms are widely distributed on growing plants and grow actively and produce acid in such situations as sauerkraut, ensilage and in plant juices such as are used in making cane sugar, maple sugar, palm sugar, etc. So much of the specific carbohydrate of *Leuconostoc* is left in commercial cane, beet, and other sugars that specific precipitin tests can be obtained by mixing a little "sugar water" with suitable immune serum.[19]

It is particularly interesting to note that the reactive substances in the cane and other commercial sugars, though apparently derived from *Leuconostoc,* and react with *Leuconostoc* antiserum, also react with type II pneumococcus antiserum. This is an example of the rather common phenomenon of the occurrence of closely related, if not absolutely identical, immunologically active substances in different species of organisms. The organisms may be rather closely related, as in *Leuconostoc* and type II pneumococcus (two kinds of Streptococceae) but sometimes they are not at all related, as in type IV pneumococci and human erythrocytes, or in the rickettsiae of typhus fever and the *Proteus* bacilli (see pages 573 and 860).

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Chapter 33

THE SPIRAL, RIGID BACTERIA. GENERA SPIRILLUM AND VIBRIO

For a long time after the observations of Leeuwenhoek, spirally twisted or curved bacteria were among the most frequently observed. This was due to the fact that they are often relatively large, often motile, occur plentifully in stagnant pond water or woodland pools, and often grow well in very simple solutions. Among these common spiral organisms are the flexible spirochetes discussed as the order Spirochaetales, and the tribe of *rigid* Spirilleae, to be discussed in this chapter.

At present six genera are recognized in this tribe: *Vibrio, Spirillum, Cellvibrio, Cellfalcicula, Desulfovibrio* and *Thiospira.*

The names of two genera are derived from their ability to digest cellulose. The names of the first two are derived from their morphology; the names of the last two genera from the fact that their metabolism involves sulfate reduction or oxidation. All are *rigid,* curved or spiral in form, non-sporeforming, gram-negative, and motile. Some are aerobic; other facultative or anaerobic. The Spirilleae are saprophytic soil and water organisms in general, although one or two dangerous pathogenic species are found in the genera *Spirillum* and *Vibrio.*

GENUS VIBRIO

This genus includes short rods which are always curved in a portion of a spiral turn (Fig. 265). Their length seldom exceeds 10 microns and their diameter 0.15 to 1 micron. They are chiefly aerobic and are motile by means of one or more polar flagella. There are numerous "species" of vibrios, many of which greatly resemble each other.[1]

The group of vibrios, as a whole, is of little importance except as scavengers living almost entirely in stagnant ponds and polluted rivers such as the Hudson and Ganges. *Vibrio comma* resembles others in living for long periods in aquatic habitats, but is a striking exception in being the cause of Asiatic cholera. Vibrios very closely related to *V. comma* and almost indistinguishable from it are sometimes found in the stools of persons ill with enteritis of more or less severity, but they have an unknown etiological relationship to such conditions.

Many of these vibrios are distinguished by place names, such as *V. gindha*, *V. danubicus* and *V. massauah*. A species possibly of real pathologic importance is called *V. proteus*. Nearly all of these vibrios are serologically different from *V. comma,* and may be distinguished by agglutination reactions and Pfeiffer's test. Immunological properties of the flagellar substances (H antigens) as contrasted with the body substances (O antigens) have been described by Burrows et al.[2] One vibrio species which probably causes a cholera-like disease is known as the El Tor vibrio. It was first found among pilgrims to Mecca who were suffering from cholera. El Tor is the name of a town on the western side of the Sinai peninsula. This vibrio is strongly hemolytic. The others are not.

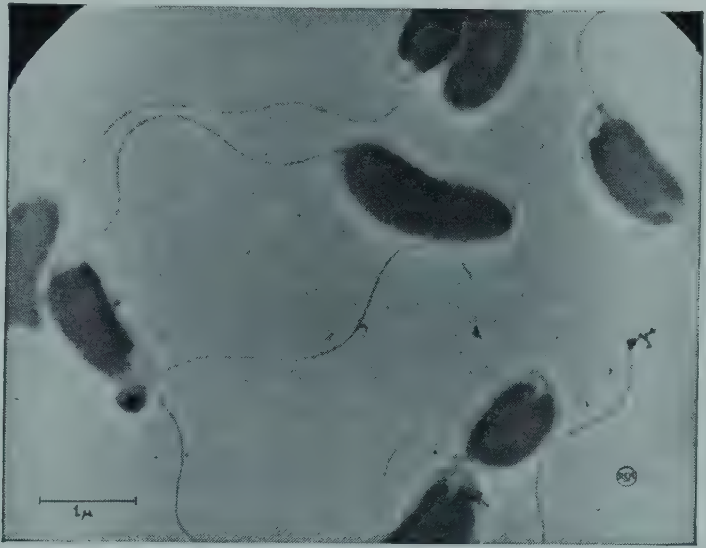

Fig. 265. Electronograph of *V. comma*. (Courtesy Dr. T. F. Anderson and Dr. K. Polevitsky.)

These miscellaneous species sometimes cause confusion in attempts to diagnose various enteric diseases bacteriologically, especially in India and the Orient. Some other vibrios, like that discovered by Metchnikoff (*V. metchnikovi*), are pathogenic for lower animals like guinea pigs and pigeons, which is not true of *V. comma*.

Isolation of Vibrios. There are three special physiological characters of most vibrios (and especially *V. comma*) upon which methods for their isolation are based. One is a strong tendency to grow at the surface of nutrient liquids in response to need for oxygen; second, a preference for, and tolerance of, an alkaline reaction (*p*H 8 to 9) which retards the growth of many of the bacteria associated with vibrios in fecal material (*e.g., Streptococcus faecalis, Escherichia coli*), and third, ability to metabolize peptone rapidly. Therefore, very rapid growth occurs at the surface of alkaline peptone* solutions, so that transfers from the surface film of such cultures after six to eight hours' incubation often yield almost pure cultures of *V. comma.*

Vibrio comma and its congeners grow well on ordinary laboratory media also. The colonies on agar are small, colorless and translucent. Most vibrios are markedly proteolytic, liquefying gelatin and digesting casein, as well as being active in the decomposition of carbohydrates like dextrose and sucrose, and alcohols such as mannitol. *V. comma* actively digests starch and, under good conditions of aeration, also attacks the organic acids resulting from starch metabolism so that the final end products are mainly CO_2 and H_2O.[3, 4] This is of importance in making vaccines.[5] Lactose is not hydrolyzed by *V. comma* in which respect it resembles the pathogenic Enterobacteriaceae such as the typhoid, paratyphoid and most dysentery bacilli.

The Cholera-red Reaction. When concentrated sulfuric acid is added to a peptone solution containing sodium nitrite and indol, a cherry-red compound is formed. This is called the *nitroso-indol reaction.* Any organism which, in simple peptone broth, reduces nitrates to nitrites and *at the same time produces indol,* will give the nitroso-indol reaction when sulfuric acid is added to the culture. *Vibrio comma* exhibits the phenomenon and, as the reaction was at one time thought to be especially characteristic of this organism, it was spoken of as the "cholera-red" reaction. It has since been found that a number of other vibrios have the same property. Not all strains[1] of *V. comma* exhibit the reaction, and some are variable.

Pfeiffer's Reaction. In this test, the organisms are injected into the peritoneal cavity of an *immune* guinea pig. The peritoneum is punctured at short intervals thereafter and organisms withdrawn for examination. They are seen to undergo progressive lysis due to the action of the specific sensitizer and the complement in the guinea pig's blood serum. The reaction is highly specific. Normal pigs give no such reaction. This test gives a striking illustration of the general principle of specific bacteriolysis.

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* *Alkaline egg-peptone solution* (*Goldberger*):

"(*a*) Prepare an alkaline egg solution by first shaking or beating up an egg with an equal volume of water and then adding to this egg-water an equal volume of a 5 per cent solution of *anhydrous* sodium carbonate. Steam for three-quarters to one hour. (*b*) Prepare Dunham's solution: peptone 10 gm., salt 5 gm., water 1000 ml.

"For use mix (*a*) and (*b*) in proportion of 1:9. Run through paper filter; distribute in 10 ml. quantities in tubes and sterilize by steaming for one and one-half hours, after which they are ready for use. This solution is of a pale straw color. It is opaque, and a slight precipitate settles to the bottom of the tube on standing, but this interferes in no way with its serviceability. It will keep at least a week."

In emergency, ordinary 1 per cent alkaline aqueous solution of peptone may be used.

Asiatic Cholera. As indicated above, *V. comma* can live in the human intestinal tract. Many healthy carriers of *V. comma* exist in the Orient.

Like dysentery, cholera is characterized by an intense diarrhea and prostration. Studies by Burrows et al. have revealed the presence and properties of an endotoxin. It is antigenically active and stimulates potent agglutinating and protective antibodies in experimental animals. Curiously, protective anti-sera are not capable of neutralizing the endotoxins *in vitro*.[6] Whether exotoxins also occur is not certain. Great damage also is done by the dehydration of the patient which is a consequence of the diarrhea. The mucosa of the large intestine comes away in flakes and the stool, being thin and watery, is described as "rice-water stool." These flakes of mucosa contain large numbers of microscopically demonstrable cholera vibrios and the organisms may be cultivated from the stools by the use of alkaline-egg-peptone water medium as already described.

Cholera is confined today to localities where grossly polluted water is used. It is to be found chiefly in seaports and other cities of the Orient. While now rare or absent in the United States,[8] this frightful disease is an ever-present danger in the Orient, and the aid of the World Health Organization was invoked to help control an epidemic in Egypt during 1947. Data published by the World Health Organization set the total number of cases at around 7300, with about 3200 deaths, between September 23 and October 20th.[9] In Korea some 1200 cases with 650 deaths were reported in June 1946.[10]

Cholera is a classical example of what results from lack of sanitation, especially with respect to water supplies. A century or less ago, cholera was to be found in practically every large city in the world,[8] particularly in communities where there was much of a transient population or in places which were centers for religious, military or other concentrations of large numbers of people, with no effective sanitary provisions with regard to sewage and pollution of water and food.

During the 1947 epidemic in Egypt pilgrimages to holy cities from the epidemic area were stopped. Other measures used in attempts to control the spread of the epidemic are listed in the literature cited.

Cholera Vaccines. Although endotoxins of *V. comma* capable of stimulating active immunity to the disease have been described,[2, 7] current practice in preparing vaccines is to use whole organisms, cultivated in a harmless nutrient solution or suspended in saline, and killed by heat or chemicals such as phenyl mercuric acetate.[5]

Methods of preparing vaccine vary greatly. In the system of Jennings and Linton aeration is an important feature.[3] However, when the Egyptian Government requested aid in the form of cholera vaccine in 1947, so many widely differing types of material were received from various countries that the World Health Organization appointed a committee to undertake the establishment of an international standard.

Burrows and his associates have studied immunity to cholera in guinea pigs. These workers report that the infection in guinea pigs simulates the natural disease in man. A little known phenomenon of immunity was observed. Antibodies (agglutinins and protective substances) were found in the intestinal contents. These were designated copro (feces) antibodies.[7] Immunity, in previously infected or vaccinated animals, appeared to be more closely related to coproantibodies than to serum antibodies. Coproantibodies appear to be independent of serum antibodies. Urinary antibodies also occur.[7]

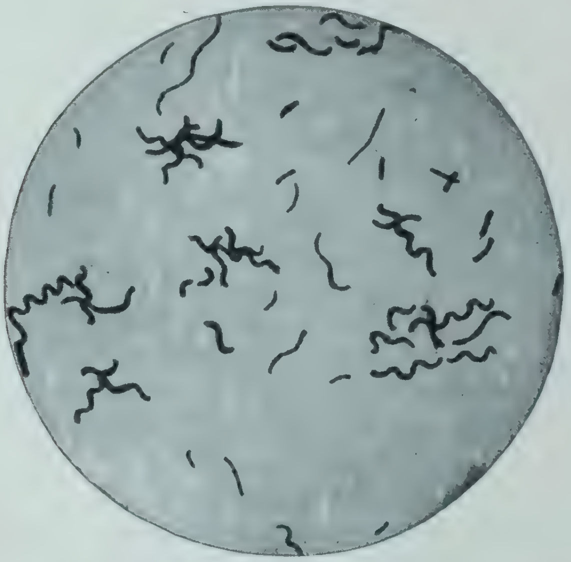

Fig. 266. *Spirillum rubrum* (× 900).

Similar antibodies have been found in human volunteers inoculated with cholera vaccine, and typhoid and dysentery coproantibodies have appeared following injection of typhoid and dysentery vaccines. It seems likely that coproantibodies may be of special significance in diseases like cholera and dysentery in which the antibodies have no opportunity to act upon the organisms in the blood and tissues, since dysentery and cholera organisms do not invade blood and tissues.

GENUS SPIRILLUM

This ge contains no species known to be of special interest to the ind alist, or the agriculturalist and only one or two of minor medical i est. All but the last mentioned are harmless saprophytes and scav rs, living in stagnant or polluted water and putrefying materials

The s lla are larger than many true bacteria, ranging from 1 to 50 m ons in length though only 0.5 to 3 microns in diameter.

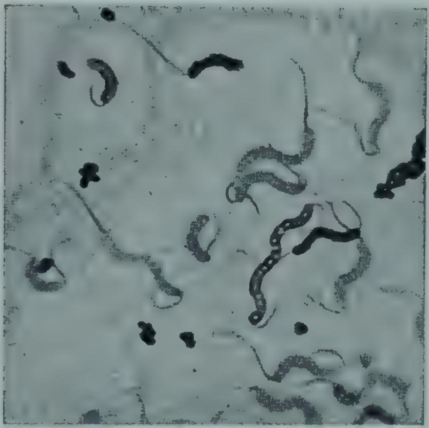

Fig 67. Typical spirilla showing flagella. × 1000. Note granules. (Myers, J. Bact., vol. 40.)

T y are spirally twisted through 1 to 2 or 3 complete turns (Fig. 2) and are motile by means of polar flagella (Fig. 267). They a gram-negative and produce no spores, stalks, branches, sheaths or d osits of elements like sulfur or iron. They usually grow with dif ulty on initial isolation, and their purification by ordinary procedures not easy. Perhaps this is one reason why they have not been more lly studied. Myers[11] has contrived to cultivate them in pure culture y first preparing infusions of stagnant water, dung or sewage enriched ith peptone, meat or fish. After a few days at room temperatures he fluid usually swarms with many sorts of microorganisms.

Ordinary nutrient agar does not serve well for initial isolation from such cultures. If the fluid from an infusion such as the above be sterilized by passage through a Seitz filter and solidified with 2 per cent agar in Petri dishes, colonies of spirillum can be obtained by inoculation with the unfiltered infusion.

After initial isolation growth on ordinary medium is relatively easy. The author maintains a strain of *Spirillum* (sp?) on blood infusion agar from year to year. These organisms and many others have also been maintained in a state of "suspended animation" desiccated in a vacuum for several years.[12]

Spirillum volutans, one of the largest species, is of interest because of its metachromatic granules, from which the term *volutin,* in reference to the species, is derived.[13] The motility and form of these spiral organisms is well demonstrated in hanging-drop preparations made from cultures in peptone-tap-water solution and incubated four days at room temperature. Negative staining is an effective means of making the organisms' arrangement visible. Beautiful demonstrations may also be made with the darkfield apparatus.

Spirillum Minus and Rat-Bite Fever (Sodoku). *Spirillum minus,* classified with the spirilla because of its two to four spirals, its rigidity and its polar flagella, is often thought and written of by medical bacteriologists as a spirochete because (*a*) it causes in man[14] a disease (rat bite fever or sodoku—from Japanese *so* = rat; *doku* = poison) having several of the fundamental clinical features of a typical spirochetal disease (syphilis); (*b*) because, like several of the true spirochetes, it has never been successfully cultivated; and (*c*) because it is rather refractory to ordinary stains. It is gram-negative.

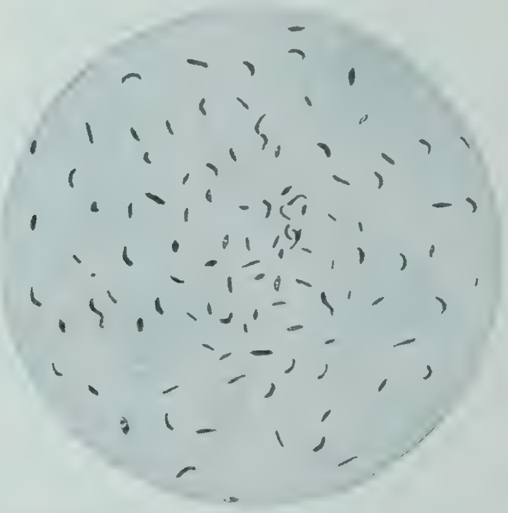

Fig. 268. *Cellfalcicula viridis* (× 900).

Sp. minus occurs in the blood of rats and possibly other animals and is transmitted from them to each other and to man by their bites. The organism is rarely found in the normal mouths of rodents but their injured and bleeding gums can contaminate their bites with blood. At the site of the bite of rats in human beings an encrusted ulcer develops, which is analogous to the chancre in syphilis and sometimes, like the latter, contains numerous spirilla (and often streptococci, etc.) demonstrable by darkfield examination and by injection of scrapings i ice. Later, fever of a relapsing type is common and a rash app roughly analogous to the rash in secondary syphilis. The Wass n reaction for syphilis varies but is said to be positive in about h some groups of cases of rate-bite fever. The infection responds ly to treatment with drugs like arsphenamine, which are speci r syphilis.

Inocul of patients' blood or of pus from swollen glands or the chancre- lcer into mice and guinea pigs usually results in an infection of t imals, and the organisms can be found in their blood or peritone id. Since laboratory mice and guinea pigs, as well as wild rat en harbor *Sp. minus* naturally, only animals known to be free of nfection should be used for diagnostic purposes and they should egregated from other animals so as to avoid infection of each o y bites. The mice may show no signs of illness after injection bu organisms appear in their blood. Blood should be examined at sho ervals for a month before they are discarded as negative (see also s n on *Streptobacillus moniliformis*).

GENERA CELLVIBRIO AND CELLFALCICULA

T rganisms in these genera are quite similar to each other, the cells he former genus being usually long, plump, curved rods with roun ends, those of the latter being relatively short (less than 2 μ), with inted ends (Fig. 268). All are motile, gram-negative, without spo aerobic and facultative. They inhabit, especially, soils contai much decaying vegetable matter and are active in cellulose de position. *Cellvibrio* grow best in the absence of organic matter ex cellulose, although some few species may be induced to grow on ptone agar, or agar with various carbohydrates. *Cellfalcicula,* h ver, are specific as to cellulose metabolism and appear unable t ilize other sources of carbon.

acteria of the genus Desulfovibrio are of importance in the sulfur c e, in maintaining soil fertility, and in other beneficent or nefarious ks, and have been discussed in connection with the sulfur bac a (see page 445).

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Chapter 34

THE FAMILY ENTEROBACTERIACEAE. THE MICROBIOLOGY OF WATER

The family Enterobacteriaceae is made up of five tribes and nine genera of closely related and morphologically identical* bacteria subdivided into scores of species and serological types. The beginning student need not be discouraged by this, however, as many of these are rare, illy defined, almost identical or of doubtful or unknown significance and need not be included in this discussion. Here we shall deal only with major groups and species of particular interest.

The family includes only gram-negative, non-sporeforming, facultative, morphologically simple, non-branching rods, with simple growth requirements. Most of these usually inhabit or at times occur in, the intestinal tract; hence, Enterobacteriaceae. The distinctively enteric organism of this family are included in six genera: *Escherichia, Aerobacter, Paracolobactrum, Proteus, Salmonella* and *Shigella. Paracolobactrum, Salmonella* and *Shigella* are pathogens in man and animals and are discussed more fully in Section IV of this book. Also included in the family Enterobacteriaceae are: one genus of rods much like *Aerobacter* but found mainly associated with the respiratory tract (*Klebsiella*); and two genera of rods, also much like *Aerobacter* or *Escherichia,* but which rarely or never occur in the intestinal tract. These two genera, *Erwinia* and *Serratia,* are common in soil and on plants; the *Erwinia* cause plant diseases (see Chapter 40).

The Enterobacteriaceae are fairly uniform in size and shape, being characteristically cylindrical, with a diameter of from 0.8 to 1.5 microns and varying in length from about 2 to 12 μ, depending on age of culture, environmental conditions and other factors. All will grow well at temperatures of from 20° to 40° C., and at a *p*H of from 6.5 to 8.0, on media such as simple peptone solutions, extract agar, or, in many cases, on simple mineral solutions with an organic source of carbon such as glucose. The Enterobacteriaceae, as a group, are rather active in fermenting various carbohydrates and attacking organic compounds such as tartrates, aldehydes, amino acids, etc. A few species are proteolytic. Some are motile, some are not.

Many of the Enterobacteriaceae are somewhat variable in biochemical properties and this has made taxonomy of the group difficult. An excellent discussion of this phase of the subject has been given by Borman, Stuart and Wheeler.[1] These authors divide the family into two parts as shown in the following table:

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* Except that some have flagella, others not.

TABLE XIII

THE ENTEROBACTERIACEAE

Pigment not produced		Pigment produced
Lactose fermented promptly	*Escherichia* *Aerobacter* *Klebsiella*	*Serratia* (Blood red pigment on starchy media)
Lactose fermented slowly or no gas	*Paracolobactrum* *Erwinia* (no gas; proteolytic)	
Lactose not fermented	*Salmonella* *Shigella* *Proteus* (decomposes urea; proteolytic)	

The groups overlap somewhat since many species or variants of each species provide connecting links between the groups listed. What one actually finds is a series of groups of organisms which have properties merging into those of neighboring groups, to form a sort of microbial spectrum where borderlines of distinction are often hazy. An example is seen in the relationship of the *Erwinia* to the rapid lactose fermentors.[2]

Since many Enterobacteriaceae of little or no pathogenic significance are so commonly and widely distributed in soil, water, air, milk, foods, etc., it is convenient to combine more detailed discussion of those (*Escherichia, Aerobacter, Proteus, Erwinia, Serratia*) with a discussion of their general relationships in the chapters on microbiology of soil, water, air, etc., to follow in this section. The pathogens (*Salmonella, Shigella, Paracolobactrum*) are discussed elsewhere.

THE MICROBIOLOGY OF WATER

The microbial flora of water depends to a great extent on: (1) the kinds of substances in the water which may serve as pabulum, *e.g.*, iron for "iron bacteria," H_2S for "sulfur bacteria," organic matter as dung or sewage for saprophytic bacteria, and so on. (2) The presence of substances unfavorable to certain organisms. For example, sea water is too saline for many species; H_2S is unfavorable to many strictly aerobic forms as it poisons cytochrome oxidase; acids, as H_2SO_4 from *Thiobacillus*, or from industrial wastes, are unfavorable to many organisms. Many streams contain a certain amount of organic and inorganic materials which favor some organisms and are unfavorable to others. (3) The microbial flora depends partly on the presence and numbers of other living organisms, especially protozoa, which consume billions of bacteria. (4) The microbiology of water also depends largely on the sources from which organisms are contributed.

Fresh surface water cannot be said to have any characteristic bacterial flora, the kinds of bacteria in it depending on its mineral and organic content, the soil with which it is in contact, surface pollution and numerous other factors. Many of the "higher bacteria" (Section II) are common in fresh water lakes, etc. Rainfall increases the numbers of soil bacteria in the waters into which it drains. The lower Hudson River has a flora representative of heavy sewage pollution; *Escherichia coli* and other Enterobacteriaceae as well as enterococci and *Clostridium perfringens* are present in large numbers. The soil saprophytes like *Spirillium, Sarcina, Micrococcus, Mycobacterium, Bacillus,* yeasts, molds, Actinomycetes, and many others also find the organic fecal matter good pabulum. The nature and extent of the bacterial populations in meadow streams where cattle wade will obviously differ from those in high mountain torrents derived from melting snow.

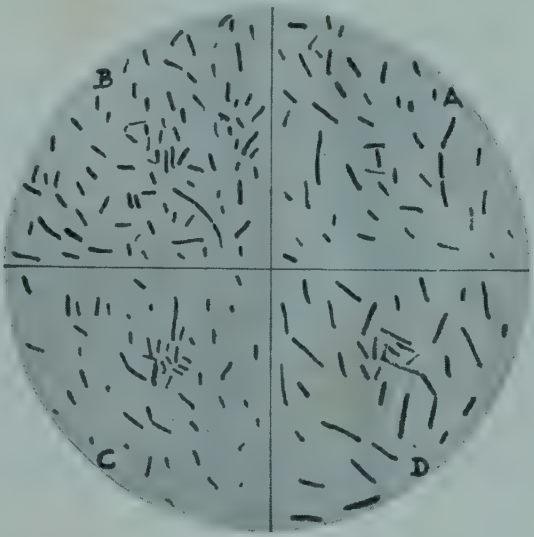

Fig. 269. Four common species of soil bacteria: *A, Chromobacterium violaceum; B, Achromobacter liquefaciens; C, Flavobacterium aquatile; D, Alcaligenes faecalis* (× 900).

Many of the common soil organisms often found in fresh surface waters resemble the Enterobacteriaceae in several respects. There is a large group of gram-negative rods, resembling the Enterobacteriaceae morphologically, rarely or never found in the intestinal tract, which live in soil and water as saprophytes. They are classified in various families and genera depending on various characteristics such as violet or yellow pigment formation (*Chromobacterium, Flavobacterium*), pathogenesis in plants (*Agrobacterium, Xanthomonas*) and so on. Some attack few of the organic substances usually tested, such as glucose and gelatin (Family Achromobacteriaceae), others are metabolically active genera such as *Pseudomonas*. Members of the genus *Alcaligenes* (especially *A. faecalis*), morphologically like Enterobacteriaceae, are also often found in water. These are sometimes found in the human intestinal canal, possibly associated with pathological conditions there. They are closely related to the genus *Rhizobium* (see page 605). The *Alcaligenes, Flavobacterium, Chromobacterium* and *Achromobacter* resemble each other morphologically (Fig. 269) and in lacking acid-producing powers (although often *utilizing* simple sugars without acid formation). Some are proteolytic and many are motile. Occasionally species give trouble in the dairy by causing spoilage of ice cream (*Achr. lipidis*), or "ropy" milk or cream (*Al. viscosus*, Fig. 270).

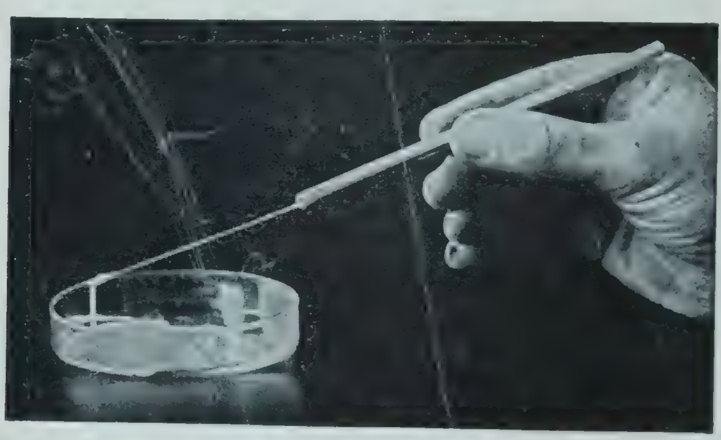

Fig. 270. Ropy cream. Causative organism *Alcaligenes viscosus*. (Reprinted by permission from Hammer, "Dairy Bacteriology," John Wiley & Sons, Inc., publishers.)

Chromobacterium violaceum, the type species of the genus *Chromobacterium*, is of interest because of its violet pigment which has antibiotic properties. *Flavobacterium synxanthum* sometimes grows in milk at about 30° C., giving it a yellow color ("yellow milk") and is therefore of interest to dairy bacteriologists. In general, however, these genera are of lesser importance.

GENUS PSEUDOMONAS

The *Pseudomonas* are classified in the family Pseudomonadaceae, which are distinguished by polar flagella in contrast to the peritrichous flagella of the Enterobacteriaceae which they otherwise much resemble. *Pseudomonas* are very commonly encountered in water, soil, putrefying materials, etc., along with the Enterobacteriaceae. Common species are *P. fluorescens* and *P. aeruginosa*. Many are actively proteolytic. A number of species live in the sea and are photogenic.

Pseudomonas fluorescens is present in all fertile soils and surface waters. It produces a greenish-yellow fluorescent pigment in cultures containing organic nitrogen (peptone).[4]

Pseudomonas aeruginosa, type-species of the genus, is of special interest. It is distinguished from other *Pseudomonas* by the production, in addition to the yellow-green pigment, of a turquoise-blue pigment, *pyocyanin,* which may be extracted from broth culture with chloroform. This organism is not infrequently found in wounds or ulcers which have not healed promptly. It causes the pus in such situations to turn a greenish-blue color, hence its older name (*pyo* = pus; *cyaneus* = blue). It probably has not the power of invading healthy tissues but sometimes gains a foothold and grows in already diseased foci, living a saprophytic existence there, possibly producing substances which result in the death and destruction of already injured tissue cells. It takes advantage of such opportunities for infection and is another example of bacterial opportunist. An outbreak of diarrhea in adults and among new-born children was caused by this organism.[5] Elrod and Braun have shown *Ps. aeruginosa* to be able to cause a leaf-rot disease in tobacco and lettuce.[6] *Ps. aeruginosa* also causes a fatal disease in poultry.

Blue Milk. The organism most commonly responsible for this curious condition is closely related to *Pseudomonas aeruginosa* and is called *Ps. syncyanea.*[7] It resembles *Ps. aeruginosa* in many respects and gains entrance to the milk from soil.

The growth of this bacterium in milk which has become slightly acid (due to the simultaneous growth of *Streptococcus lactis*) results in a deep blue color. The pyocyanin-like pigment acts in the manner of an acid indicator, for it is colorless or brownish in fresh milk. The phenomenon of "blue milk" is typical of discolorations of milk and other products produced by pigmented microorganisms.

GENUS PROTEUS

This genus comprises a group of soil and water saprophytes, common in decaying animal or vegetable matter and often found in the human intestine or as opportunists in infections (especially cystitis) of man and causing diseases in lower animals.[8, 9, 10] These are also situations in which species of *Pseudomonas* are sometimes found.

The *Proteus* species, while grouped with the Enterobacteriaceae because of their morphology, staining, ability to grow in the intestinal tract and their fermentative powers, also resemble the *Pseudomonas* in many respects, but do not produce pigment. *Pseudomonas* have polar flagella, while *Proteus* have peritrichous flagella,* a stable and fundamental differential character. *Proteus* is a facultatively anaerobic group, while *Pseudomonas* are aerobic. The power to hydrolize urea is another distinctive character of *Proteus*.[11]

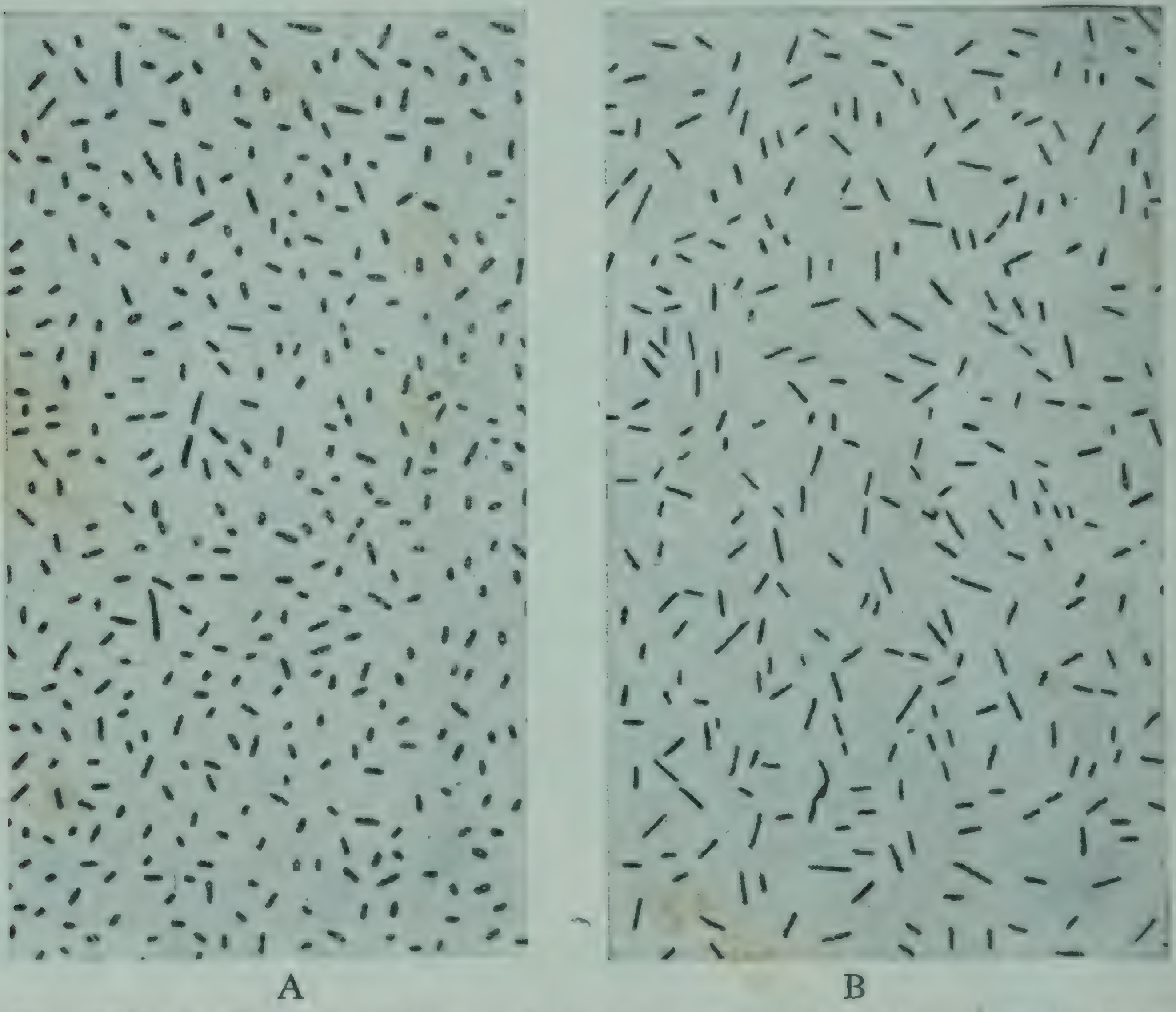

Fig. 271. *A*, Proteus vulgaris, plain agar, twenty-four hours, stained with gentian violet. *B*. Pseudomonas fluorescens, plain agar, twenty hours, stained with gentian violet. (Ford, Textbook of Bacteriology, W. B. Saunders Co.)

Proteus colonies are usually distinctive in that they spread rapidly over the surface of solid media (see "swarming," pages 172, 419), forming a thin, gray, almost transparent film which may escape notice entirely unless it is especially sought.† However, an aflagellate nonmotile variant form also occurs. In the aflagellate phase of variation, the colonies of *Proteus* are small, discrete and circular. As will be seen later, these aflagellate variants of *Proteus* have a peculiar interest in connection with the diagnosis of Rocky Mountain spotted fever and related diseases, being agglutinated by the serum of patients with these infections, without, however, being in any way related to the cause of those diseases. *Proteus* has been occasionally implicated in outbreaks of gastroenteritis.[13]

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* Except nonmotile variants.

† Certain members of Enterobacteriaceae may exhibit this property under suitable conditions, while motility may be developed in some species of the ordinarily nonmotile *Shigella* under certain conditions.[12]

GENUS SERRATIA

Serratia marcescens, type species of the genus *Serratia,* is like *Aerobacter* in many ways (see below) but produces a blood-red pigment, especially when growing upon starchy foods at room temperature. It is a common, facultative, motile, saprophytic rod of the soil and water and occasionally is found in milk. It is not found in the intestine. Due to its rapid growth and red coloration and digestion and liquefaction of the substrate in the presence of adequate moisture, it can produce an intensely blood-red pasty condition of bread. It was named *Serratia* for a famous Italian physicist, Serrati. The name *marcescens* indicates its hydrolytic activity. It is of industrial importance as a cause of spoilage in improperly stored milk, producing its red pigment and "red milk."

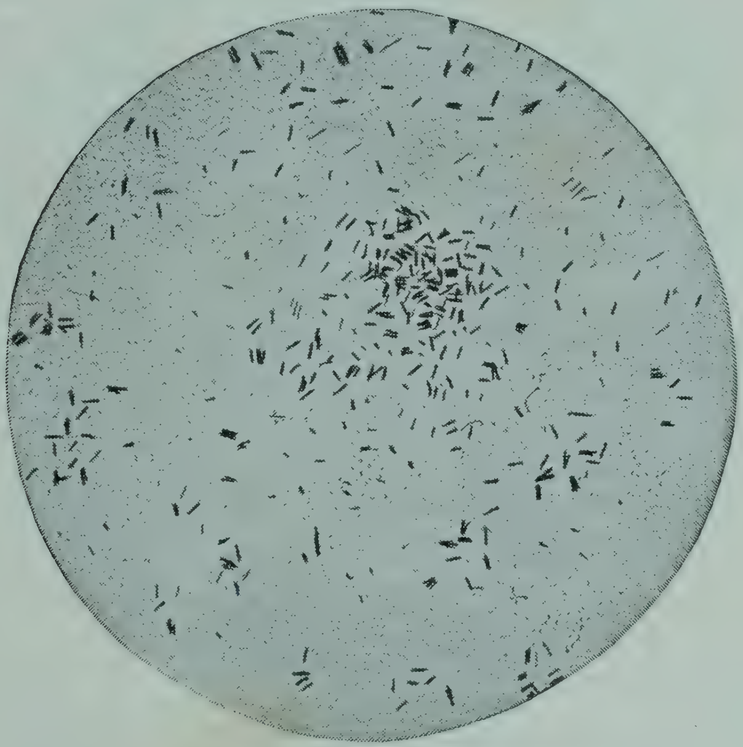

Fig. 272. *Serratia marcescens* (× 900).

In size, it is one of the smallest bacteria and for this reason is frequently used as a control organism in testing the efficacy or fineness of bacterial filters. Due to its rapid growth and distinctive color, it is readily detected in filtrates from defective filters (Fig. 272).

These various organisms, all gram-negative, non-sporeforming, facultative rods of soil, water and intestine, constitute a sort of "metabolic gradient." This has at its *upper*, pathogenically active but metabolically inactive, extreme the genus *Shigella*, the cause of dysentery, and the habitat of which is confined largely to the human intestine. The shigellas cannot live for extended periods in the soil or water, since they are accustomed to life in a human host. At the *lower* end of the hypothetical gradient we find the metabolically active genus *Aerobacter*, a group of species which are primarily soil organisms, only occasionally being found in the intestine, and lacking in pathogenicity. Between these two extremes there are *Salmonella typhosa, Paracolobactrum, Escherichia coli*, etc., which represent gradations in metabolic activity and dependence upon a host of some sort for support (parasitism).

The genus *Serratia* might be regarded as an extension of the gradient below *Aerogenes*, into the group of definitely and almost exclusively harmless soil and water types seldom or never found in the intestine—a sort of infra-*Aerogenes* group, even more active, metabolically, and more resistant to "outdoor life" than *Aerogenes*, and never found in the intestine. *S. marcescens* has many properties of the *Aerobacter*, fermenting many carbohydrates actively, being motile, liquefying gelatin, coagulating and sometimes digesting milk, producing acetyl-methyl-carbinol, carbon dioxide, hydrogen, and a variety of organic acids and alcohols from dextrose, reducing nitrates, growing well at temperatures below that of the body (25° to 30° C.) and surviving well in the soil and water. This is in marked contrast with the organisms like *Shigella dysenteriae* found at the other end of the gradient, which are much more limited in the number of substances they can metabolize, and cannot grow in the outer world and soon die off there, are sensitive to drying and sunlight, and require specially prepared organic substances for their growth (see Fig. 273).

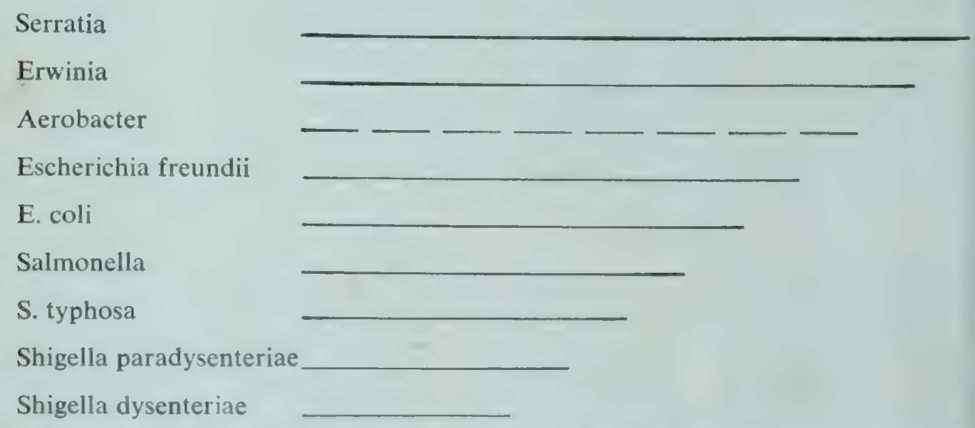

Fig. 273. Metabolic "gradient" within the family *Enterobacteriaceae*. Divisions in the line opposite *Aerobacter* may be taken to represent in order, from left to right, the ability to ferment mono-saccharides, di-saccharides, tri-saccharides, to produce acetyl-methyl-carbinol, to liquefy gelatin, etc. In other genera, the shorter the line opposite the genus, the lower the metabolic activities of that genus. When specific substances such as particular carbohydrates are used in the base line, the gradient breaks down in a few places, but the general concept that we are dealing with different degrees of specialization among the several genera still holds true. (Adapted from Stuart, New England Water Works J., *55*:355, 1941.)

GENUS AEROBACTER

The organisms of this genus possess the general characters of the Enterobacteriaceae. All are facultatively anaerobic, grow in relatively simple media and live generally in the soil and in polluted water. They occur at times in the intestines of man or lower animals. The organisms are therefore included in the rather loose term "the coliform group."

The organisms of the genus *Aerobacter* are a sort of transitional form between gram-negative, non-sporeforming facultative rods like *Proteus* and *Serratia,* living mainly in the soil, and those which have become adapted to life as a commensal in the animal intestine like *Escherichia coli*. The *Aerobacter* live in both environments, but are more commonly soil organisms than intestinal.

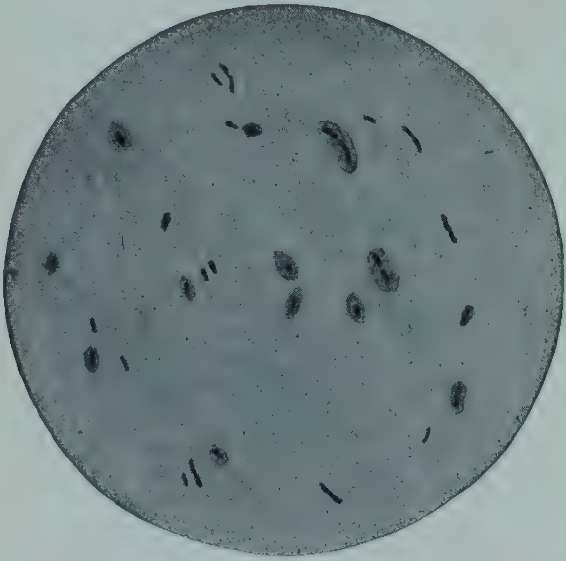

Fig. 274. *Aerobacter aerogenes* (× 900).

Aerobacter cloacae is most closely related of the *Aerobacter* to the true soil and water species. It is motile, liquefies gelatin and other proteins, is highly fermentative, produces acetyl-methyl-carbinol but does not produce pigment. It is predominantly a soil and water species like *Ps. fluorescens* or *Proteus vulgaris* but, like the latter, is occasionally found in the intestinal canal.

Aerobacter aerogenes differs from *A. cloacae* in being usually nonmotile, usually encapsulated (it produces motile, nonencapsulated variants) (Fig. 274) and in failing to liquefy gelatin. *A. aerogenes* is predominantly an environmental (or soil and water) organism, but is found in feces more frequently than *A. cloacae*.

Rake has drawn attention to an interesting relationship of *Aerobacter* to a very similar organism found as the cause of a certain type of pneumonia (*Klebsiella pneumoniae*) and to another like organism (*Donovania granulomatis*) long known as the cause of an ulcerative disease of the inguinal lymph nodes. The new studies indicate close relationship of these bacteria.[17a] Probably they should all be in the same genus—*Aerobacter.*

GENUS ESCHERICHIA

Next removed from the distinctly saprophytic and free-living forms, we find the genus *Escherichia,* a very important species of which is *Escherichia coli* (Fig. 275). This is preeminently an intestinal organism. *Esch. coli* is a little less active metabolically than *Aerobacter aerogenes* and is occasionally pathogenic. It does not form acetyl-methyl-carbinol, is not so regularly or actively motile as *A. choacae* and fails to metabolize many of the substances which can be metabolized by *A. aerogenes* and *A. cloacae.*

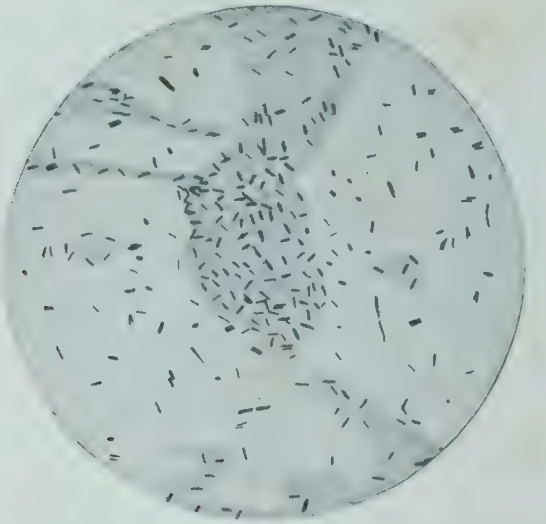

Fig. 275. *Escherichia coli* (× 900).

Escherichia freundii, a species common in the soil and water, but also frequently found in the normal human intestine,[14] occupies a position between *Esch. coli* and the *Aerobacter.* It resembles *Esch. coli* in some respects but resembles the *Aerobacter* in being able to utilize *citrates* as a sole source of energy or carbon in an otherwise wholly inorganic medium. It is often spoken of as an "intermediate" or "citrobacter."

These four species (two of *Escherichia* and two of *Aerobacter*), because of their occurrence in polluted water, have a very important bearing on the health of everyone, a fact which needs some detailed discussion.

Sanitary Relationships of the Genera Escherichia and Aerobacter. Every well-informed person knows that *Salmonella typhosa* (the typhoid bacillus) causes intestinal disease, and that typhoid fever is transmitted by food, milk, or water supplies which have become polluted with the urine or feces of a person harboring this organism. In thickly populated districts practically every body of surface water receives some fecal pollution due to the careless habits of ignorant people with respect to urination and defecation. Sometimes people may be perfectly healthy yet harbor typhoid bacilli in their intestinal or urinary tract and discharge them in large numbers, thus infecting water supplies, milk, food and other substances which they handle. Such persons are called *"carriers"* of typhoid.

Now, it might seem a simple matter to detect typhoid bacilli in water supplies, since the organisms are easily cultivated on nutrient broth or agar, but actually this is difficult for many reasons. These bacilli do not live for more than a month or so in natural water due, in part, to the antagonistic action of free-living saprophytes like *Pseudomonas aeruginosa,* and plankton organisms. Further, the numbers of typhoid bacilli in a lake or stream are usually relatively so small that, although enough might be present in a pint of water to cause disease, the practical difficulty of isolating them in pure cultures from such large amounts of fluid is great.*

Pollution of water with fecal material, whether infected or not, is obviously undesirable, both from the standpoint of danger of infection and for purely esthetic reasons. The detection in water of fecal bacteria of any kind, is, therefore, of importance in determining its suitability for drinking purposes. Two very common, readily cultivated and numerically predominant types of intestinal organism are the enterococci and *Escherichia coli.* For every typhoid bacillus there may be millions of streptococci or *Esch. coli.* Streptococci are sometimes sought in sanitary examinations of water, especially in England, but

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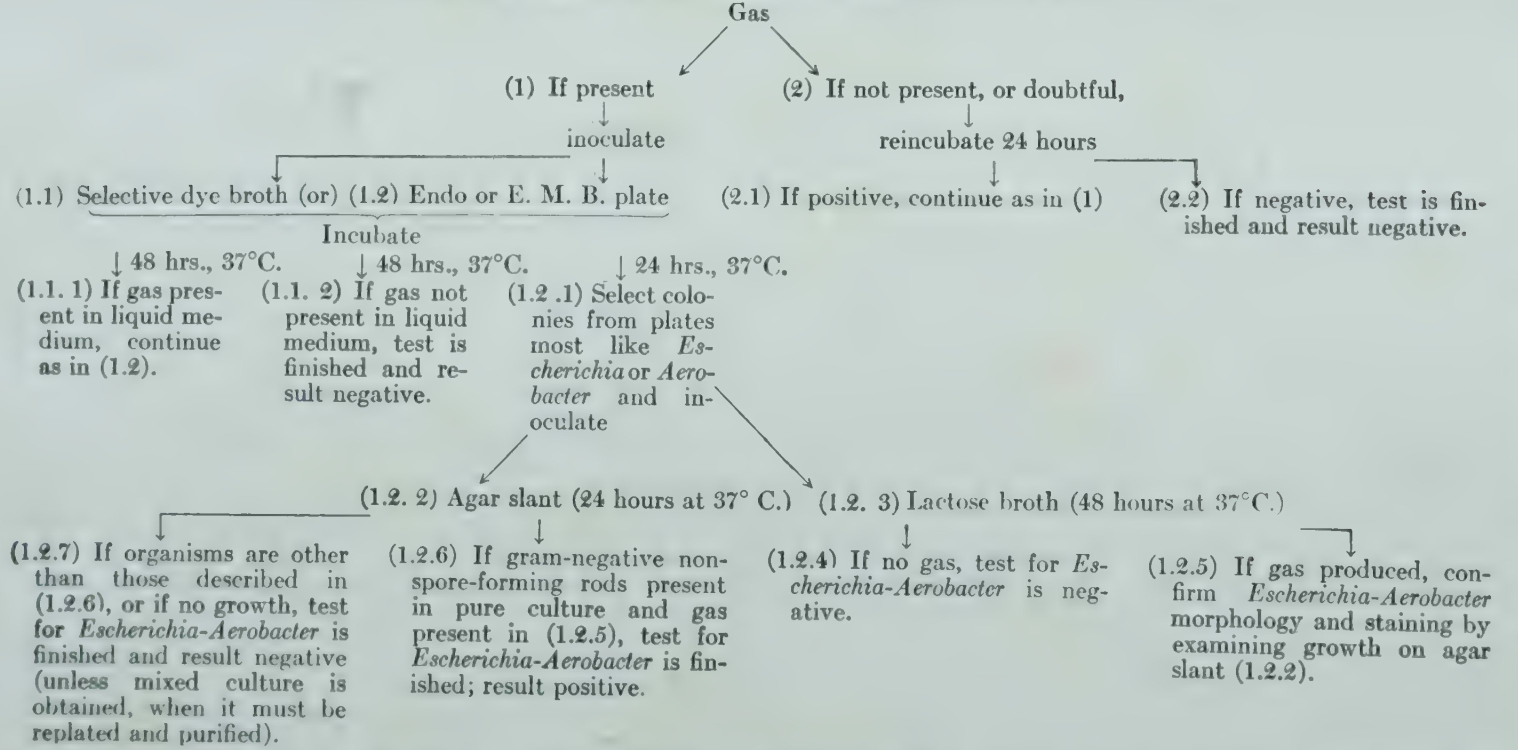
* Typhoid bacilli may be cultivated in large volumes of such water by adding to it the ingredients of a peptone medium in appropriate proportions, adding also a very small amount of *sodium selenite*† which prevents the growth of antagonistic bacteria such as *Pseudomonas* and *Esch. coli.* The medium is actually made of the already-inoculated water. It is then incubated for 12 to 14 hours at 37° C., and portions are streaked on plates of agar selective for *S. typhosa* (see page 730). After incubation of the plate colonies of *S. typhosa* may be found. Such a process is often called "selective cultivation" and is widely used in microbiology.

† Suitable medium is available from the Baltimore Biological Laboratories, Baltimore, Md.

CHART I

OUTLINE OF OFFICIAL TEST FOR ESCHERICHIA-AEROBACTER (COLIFORM) GROUPS*

0.5 per cent lactose broth fermentation tubes (24 hours at 37° C.)



Gas

(1) If present → inoculate

(1.1) Selective dye broth (or) (1.2) Endo or E. M. B. plate

Incubate

↓ 48 hrs., 37°C. (1.1. 1) If gas present in liquid medium, continue as in (1.2).

↓ 48 hrs., 37°C. (1.1. 2) If gas not present in liquid medium, test is finished and result negative.

↓ 24 hrs., 37°C. (1.2 .1) Select colonies from plates most like *Escherichia* or *Aerobacter* and inoculate

(1.2. 2) Agar slant (24 hours at 37° C.)

(1.2. 3) Lactose broth (48 hours at 37°C.)

(1.2.7) If organisms are other than those described in (1.2.6), or if no growth, test for *Escherichia-Aerobacter* is finished and result negative (unless mixed culture is obtained, when it must be replated and purified).

(1.2.6) If gram-negative non-spore-forming rods present in pure culture and gas present in (1.2.5), test for *Escherichia-Aerobacter* is finished; result positive.

(1.2.4) If no gas, test for *Escherichia-Aerobacter* is negative.

(1.2.5) If gas produced, confirm *Escherichia-Aerobacter* morphology and staining by examining growth on agar slant (1.2.2).

(2) If not present, or doubtful, → reincubate 24 hours

(2.1) If positive, continue as in (1)

(2.2) If negative, test is finished and result negative.

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* Adapted from "Standard Methods of Water Analysis," 9th Edition, 1948, American Public Health Association, New York City, N. Y.

in the United States *Esch. coli* is considered a more sensitive indicator of pollution and is more easily demonstrated.*

In order to determine the presence of *Esch. coli,* advantage is taken of its ability to ferment lactose with the production of gas. But the *Aerobacter* and *Esch. freundii* also ferment lactose with gas production and it is therefore of importance to distinguish between the three because *Esch. coli* is definitely an intestinal organism while *Aerobacter* and *Esch. freundii* are also frequently derived from soil.

SANITARY BACTERIOLOGICAL EXAMINATION OF WATER

The American Public Health Association has outlined an *official* procedure for the isolation of coliform organisms from water and has described a procedure for differentiation between *Esch. coli* of truly intestinal origin and *Esch. freundii* and the *Aerobacter.* The value of the latter differentiation for official purposes is still in debate. Any organism of the coliform group is officially (and rightly) regarded with suspicion. The details of these processes are given here in outline form only (Chart I).[17]

Briefly, the water to be examined is inoculated in measured amounts into tubes of extract broth containing 0.5 per cent lactose and incubated for twenty-four hours. If gas is formed it is regarded as *presumptive* evidence that *Esch. coli* is present.

However, the gas may be due to *Aerobacter* or some other organism such as *Clostridium perfringens,* yeasts, or synergistic combinations of microorganisms capable of producing gas from lactose. The broth culture is, therefore, either streaked on an agar medium containing some dye which inhibits all but the coliform group, *e.g.,* eosin-methylene-blue (E.M.B.) (see bacteriostasis, page 215); or transfers are made directly from the lactose broth to liquid media based on the same principle but containing no agar, *e.g.,* brilliant-green-lactose-bile broth.‡

If, after incubation of the brilliant-green-bile broth cultures, gas is produced, it is strongly suggestive of the presence of coliform organisms because most other species are inhibited by the dyes. The cultures may, for further proof, be streaked on the E.M.B. plates as mentioned above.

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* However, the use of enterococci as an indicator of fecal pollution of water has its advantages: Excellent selective methods are available;† the streptococci survive in water longer than *Esch. coli;* enterococci may be more easily identified than Esch. coli; they are not as ubiquitous as the coliform group of organisms,[15] and therefore more reliable indicators of fecal pollution.

† For example, a broth is made containing sodium azide, 0.05 per cent. This inhibits all gram-negative organisms. The culture is incubated at 45.5° C. This favors enterococci and is unfavorable to other organisms.[16]

‡ These media are available commercially.

After the E.M.B. plates are inoculated (either directly from the original lactose broth or from the brilliant-green broth), they are incubated for twenty-four hours and colonies thought to be those of coliform organisms are fished, in *pure culture,* to lactose broth for verification of lactose-fermenting properties. An agar slant is also inoculated for examination of staining and of morphology, proof of purity of the culture, *aerobic growth* and for storage. If a gram-negative, nonsporeforming rod is found, it could hardly be any organism but the coliform group, since no others would grow under the conditions provided.

The selection of coliform colonies on E.M.B. plates is facilitated by three factors: first, the plates contain lactose, readily fermented and acidified by these organisms; second, the plates contain the dyes eosin and methylene blue, which inhibit many other bacteria; third, the acidification of the lactose under the lactose-fermenting coliform colonies turns the dyes in the agar a distinctive red, blue, or purple color in and around each colony so that it is easily recognized.

This completes the official procedure and is designated as a "completed test." However, at this point we do not know more than that we have on hand organisms belonging either to the genus *Escherichia* or *Aerobacter*. For reasons already cited, additional tests are frequently made, to distinguish the *Aerobacter* from the *Escherichia,* and *Esch. coli* from *Esch. freundii* (see page 583).

Short Method for Coliform Group. A simpler and quicker procedure for demonstrating the probable presence of the coliform group in water, milk, etc., consists in direct inoculation of the sample into tubes containing medium which is highly selective for these organisms. The lactose bile-brilliant green medium already mentioned is good for this purpose. The use of buffered lactose broth containing lauryl sulfate* in 1:10,000 concentration as an inhibiting agent for extraneous organisms has given excellent results in the hands of Mallmann and Darby[18] and others, while Perry and Hajna[18a] have obtained equivalent results with 0.15 per cent bile salts as inhibitor in a medium ("E-C" medium) of similar composition.* Almost the only organisms known to be capable of producing gas in such media are those of the coliform group.

The Eijkman Test. The latter authors have shown that by making the original inoculations into tubes of buffered lactose broth containing no inhibitory agent, *but incubated at 45.5° C.,* or by making secondary transfers from original "E-C" cultures showing gas into such broth, gas-formation is obtained only in the presence of *Esch. coli* which, alone of the group, has the power to produce gas *at this temperature.*[19]

* These media are available commercially.

These procedures are excellent illustrations of the practical application of selective bacteriostatic methods, and of an incubation temperature which is critical for the activity of an enzyme (formic decarboxylase) responsible for gas production by *Esch. coli.*

Objections to these procedures are (*a*) that the selective media may be too inhibitory and thus eliminate some "weak" strains on the coliform group, and (*b*) that while the gas produced in the culture is *probably* due to these organisms, it is not proof of their presence since it is conceivable that some other species might act similarly under the conditions of growth provided.

Differentiation of Fecal and Nonfecal Types. Having obtained satisfactory pure cultures, one makes differentiations between *Esch. coli, Esch. freundii* and *Aerobacter* on the basis of indol production, the methyl red test, the Voges-Proskauer reaction and ability to utilize sodium citrate as a sole source of carbon (Table XIV). Another determination frequently made is that of the gas ratio. The test for indol has already been described. The technical details may be found in *Standard Methods of Water Analysis.*[17]

TABLE XIV

DIFFERENTIAL FINDINGS, FECAL AND NONFECAL TYPES

	Indol	Methyl Red Test	Voges-Proskauer Reaction	Citrate Utilization	Gas Ratio (CO_2/H)
Escherichia coli ..	+	+	—	—	1:1 or $CO_2 : < H$
Escherichia freundii (Intermediates or Citrobacter)	—	+	—	+	1:1 or $CO_2 : < H$
Aerobacter	—	—	+	+	2:1 or $CO_2 : > H$

Imvic Formula. A mnemonic *"IMViC"* is used, with plus and minus signs, to express a sort of formula of these differences. *I* stands for indol reaction, *M* for methyl red reaction, *V* for the acetyl-methyl-carbinol test (originated by Voges-Proskauer), *i* is for euphony, and *C* for growth in citrate broth. Thus, an organism of the coliform group designated as "IMViC + + — —" would indicate *Esch. coli* since this gives positive indol and methyl red reactions but negative Voges-Proskauer and citrate reactions.

Citrate Utilization. This test is merely an observation of whether or not growth (as evidenced by turbidity) occurs in a simple mineral solution with sodium citrate as the sole source of carbon. In order to avoid an *initial* turbidity, or the introduction of peptone from the agar slant used for inoculum (another source of organic C) the inoculation must be light.

Gas Ratio. The metabolic products evolved from dextrose by *Escherichia* and *Aerobacter* differ markedly. When *Escherichia* ferment dextrose acids and the two gases, hydrogen and carbon dioxide are formed. The amount of carbon dioxide never exceeds the volume of hydrogen, the latter usually being present in excess. The *Aerobacter* produce the same gases but *in an inverse ratio, i.e.,* carbon dioxide always predominates in the mixture.

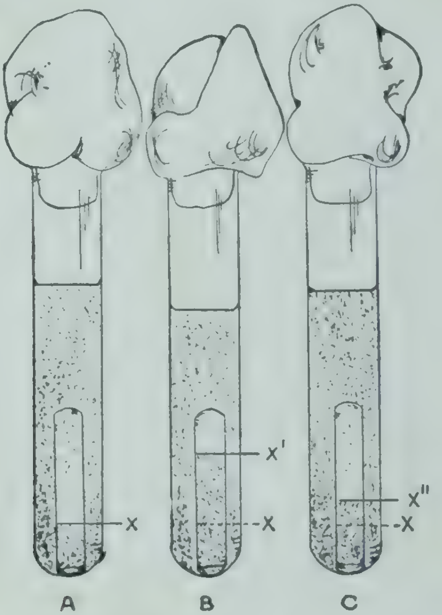

Fig. 276. Diagram showing determination of gas ratio in glucose broth in Durham tubes. *A*, Total volume of gas mixture after 24 hours at 37° C.; any coliform organism. X indicates height of fluid in inner tube before adding NaOH; *B*, *Aerobacter*. After adding NaOH, fluid has risen from X to X^1, showing the volume of CO_2 absorbed by the NaOH. The ratio of CO_2 to unabsorbed gas (H) is approximately $\frac{CO_2}{H} = \frac{2}{1}$; *C*, *Escherichia*. After adding NaOH to a tube in which the gas ratio is approximately $\frac{CO_2}{H} = \frac{1}{>1}$ or $\frac{.1}{2}$.

Acetyl-methyl-carbinol (*The Voges-Proskauer Reaction*). When dextrose is fermented by *Aerobacter* and some other organisms, such as *Serratia marcescens,* not only are acids and gases formed, but also a substance called *acetyl-methyl-carbinol* (see Chapter 15). This may be detected by adding strong sodium hydroxide to the culture and aerating well. The acetyl-methyl-carbinol undergoes oxidative changes to diacetyl and forms a compound with peptone having a salmon, pink, or red color. This is a positive *Voges-Proskauer reaction*. The *Escherichia* never form acetyl-methyl-carbinol.

One method* for detecting the ability of bacteria to form this substance exemplifies a useful principle in bacteriological technic. Here, instead of waiting several days for large numbers of bacteria to grow and accumulate in the culture and to act upon the substrate, a very large, active, young, bacterial population *already formed* is added to the dextrose broth and only a few hours are necessary for these billions of active cells to produce sufficient acetyl-methyl-carbinol to give a strong test. The method is of interest not only because of the saving in time but because it is applicable to many tests of a similar nature with other species and other substances, for example, nitrate reduction, hydrolysis of various compounds, etc. It is used in some commercial processes.

Methyl-red Test (*Clark and Lubs Reaction*). Both *Escherichia* and *Aerobacter* produce acids and gases when dextrose is fermented. *Escherichia* cultures remain strongly acid for several days and methyl red (an acid-alkali indicator) takes a definite red color (indicating acid) when added to the culture after four or five days' incubation. This is a *positive methyl-red test*. The *Aerobacter,* on the other hand, are able to attack the acids which they form from the dextrose, converting them, after four or five days, into carbonates or other nonacid substances. The medium is then found to have reverted toward a neutral or alkaline reaction and gives a yellow color (indicating relative alkalinity) when methyl red is added. This is a *negative methyl red test*.

There are many other tests for differentiation between *Escherichia* and *Aerobacter*. For example, *Aerobacter* can attack substances, such as sodium hippurate, cellobiose and various organic acids, which *Escherichia* cannot metabolize.

GENUS PARACOLOBACTRUM

This group, discussed more fully in the section on enteric disease (see page 729), resembles the *Aerobacter* and *Escherichia* in most respects but differs especially in very slow fermentation of lactose and in virulence.[21, 22, 23, 24] These slow-lactose fermenters have long confused sanitary microbiologists. They are now regarded as a sort of "connecting link" between the saprophytic coliform group and organisms of the typhoid-dysentery group. They are more fully discussed in Chapter 41 with the pathogenic Enterobacteriaceae.

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* *Rapid test for acetyl-methyl-carbinol production* (*Voges-Proskauer Test*), Coblentz, J. M.[20]

Inoculate 5 ml. of 1 per cent dextrose infusion-broth with a massive inoculum (2 to 3 mm.) from a 12-hour infusion-agar slant of the strain to be tested. Incubate at 30° C. for 6 hours. (Satisfactory results have also been obtained at 37° C.) At end of the incubation transfer 2 ml. of the broth culture into a culture tube 18 mm. in diameter and add 0.6 ml. of alphanaphthol solution (5 gm. of alphanaphthol in 100 ml. 95 per cent ethyl alcohol). Shake slightly to mix and add 0.2 ml. of 40 per cent KOH to which has been added 0.3 per cent creatine. Shake the tubes vigorously for 30 seconds to 1 minute.

A positive reaction is characterized by an intense pink-rose coloration developing in a few seconds to ten minutes after reagents are added.

SANITATION OF DRINKING WATER

The diseases most frequently transmitted by drinking water are typhoid fever and cholera. They are diseases of the intestine and are transmitted by any material, especially milk, water, or food, contaminated with infected feces.

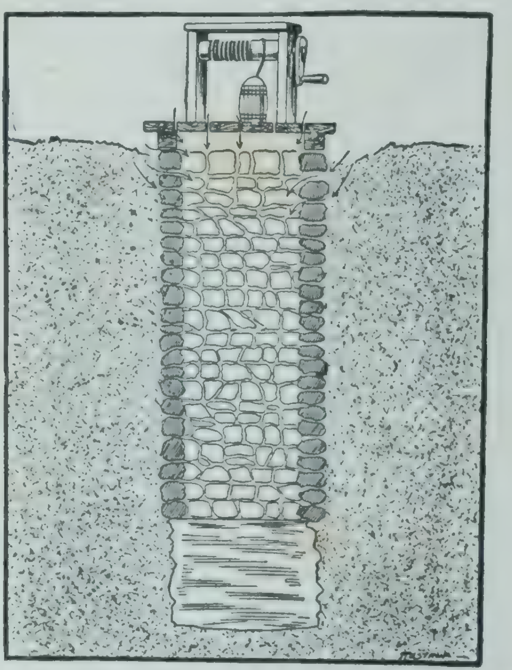

Fig. 277. Usual method of pollution and even infection of well. The surface washings may contain the feces of a careless typhoid carrier who has defecated nearby. Such instances are numerous. (Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

Bodies of water are convenient places for the disposal of sewage and other refuse and it was not uncommon, until even as late as the latter part of the nineteenth century, for a city to pour its sewage into a body of water at one point and build the intake for a piped water supply not far away. Consequently, sewage got into the drinking water and epidemics of intestinal infection, especially typhoid and cholera, occurred.

Springs, streams, and lakes also become polluted through the drainage or seepage into them of infected surface washings. Today, we know how to prevent pollution of small bodies of water used for drinking. Figures 277, 278, 279 and 280 show how wells may become polluted and how to construct wells to avoid pollution.

In dealing with large bodies of water, however, pollution is difficult to avoid at all times. The result is that such waters, if used for city water supplies, must be treated so that even occasional pollution with its dangers of infection may be guarded against.[17] The water is therefore collected in reservoirs and then piped to some sort of plant where it is usually filtered, especially if not perfectly clear and clean all the year round. In any case, it must always be disinfected with chlorine, or live typhoid bacilli may at some time get into the water pipes and kill hundreds of people. This has happened repeatedly in the past. The small amount of chlorine necessary for this purpose is absolutely harmless even though it may at times give a "chemical

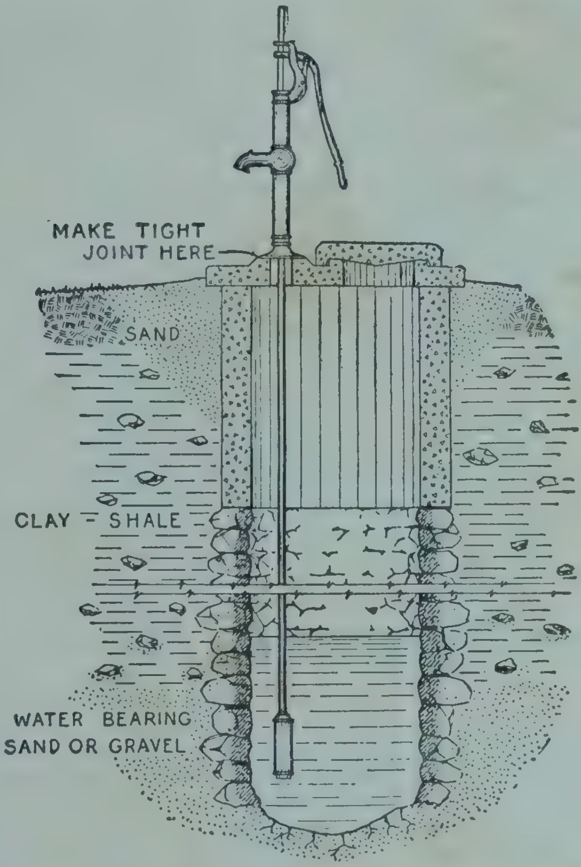

Fig. 278. Showing how a well can be protected against pollution. Note the drainage away from the well and the water-tight curb and platform. (Minn. State Board of Health.)

flavor" to the water. Modern methods of water treatment, involving "break-point" chlorination, can avoid these tastes and odors to a great extent.[25]

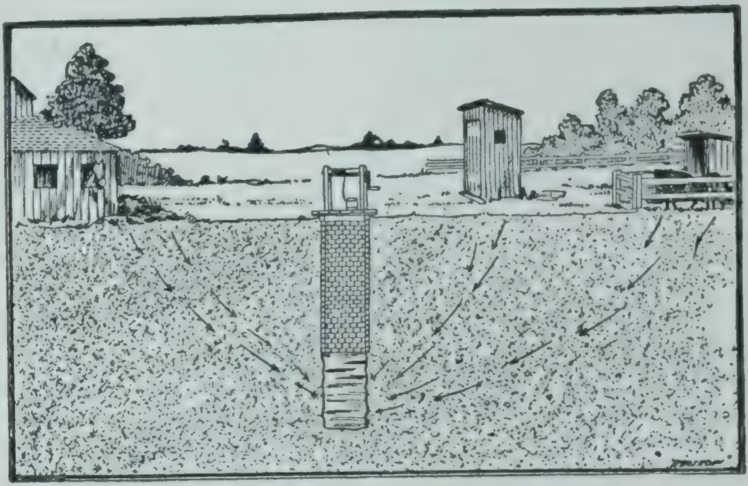

Fig. 279. Popular idea of how wells become infected from surface pollution. This probably rarely takes place in rural districts, as the soil can usually hold back most of the impurities. The danger is great, however, where fissures, cracks, or crevices exist, or where sewage enters beneath the surface of the soil from broken drains or leaky privies, especially in limestone formations. (Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

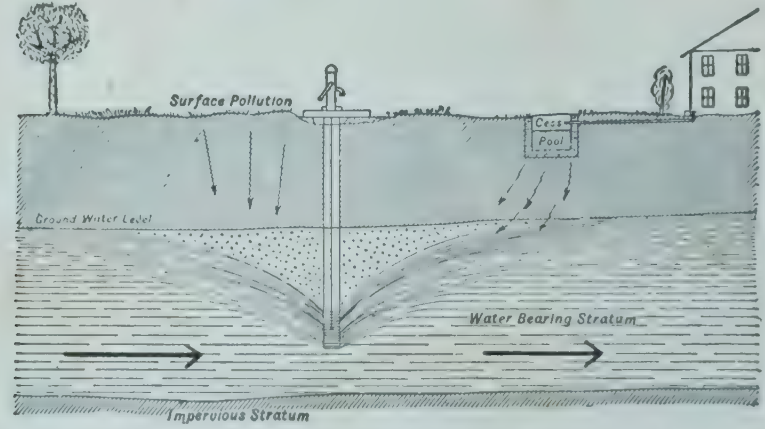

Fig. 280. Depression of the ground water level by pumping and tendency to draw nearby pollution from the soil or cesspool against the normal direction of flow. (Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

Filter Plants. *The Slow Sand Filter.* Filter plants are of two main types: the slow sand filter and the rapid sand filter. Both imitate natural processes of water purification by allowing the water to seep through clean soil, or sand, the water collecting in underdrains and being piped to cisterns where it receives a dose of chlorine.

In the *slow sand process,* large sand and gravel beds are built up over drain pipes, starting with coarse gravel at the bottom and graduating in size to rather fine sand at the top (Fig. 281). The water is led onto the sand and allowed to trickle slowly through. The area of the slow sand filters is necessarily large because the water passes slowly through it. As filteration proceeds, day after day, there accumulates, especially in the upper layer of sand, a slimy or gelatinous film composed of millions of bacteria and protozoa, which slowly closes up the pores in the sand. This makes the filter bed more and more effective but also causes the rate of filtration to become slower and slower.

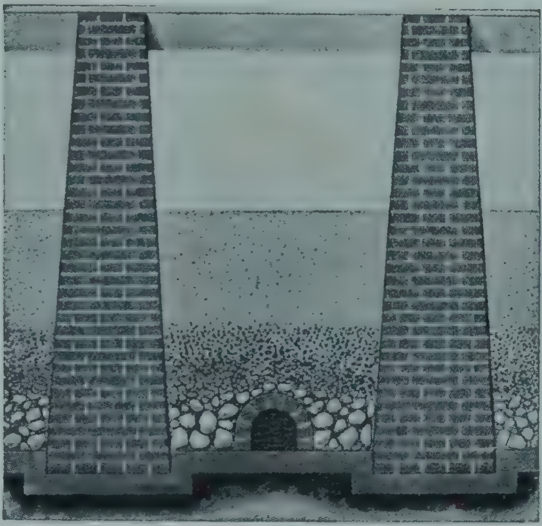

Fig. 281. A slow sand filter. (From Löhnis and Fred, "Agricultural Bacteriology," McGraw-Hill Book Co., Inc., publishers.)

This film is called the "Schmutzdecke" (German for "dirt-cover"). Through the action of enzymes, biological oxidation and reduction processes, and the ingestion of bacteria by myriads of protozoa inhabiting the slimy film, the bacterial and chemical quality of the water is greatly improved, the number of bacteria present in the filtered water becoming smaller and smaller as the action of the Schmutzdecke increases. When the gelatinous film finally becomes too thick, the filter is thrown out of service and the Schmutzdecke removed. A newly cleaned filter is not highly effective until the Schmutzdecke begins to form. The effectiveness of the filtration is constantly tested by bacteriologists in the plant who determine the numbers and kinds of bacteria present in the water during different stages of the filtration process, as well as in the finished product.

The Rapid Sand Filter. The rapid sand filter is very similar to the slow sand filter in principle, but its area is much less and it does not depend on the growth of a Schmutzdecke. It filters water much more rapidly per unit of filter-bed area. In order to obtain rapid filtration of the water it is necessary that most of the foreign material in the water be first removed by some other means. This is usually accomplished by *settling* and *coagulation.* Coagulative substances, usually ammonium aluminum sulfate, or a mixture of ferrous sulfate and lime, are added to the water. These form bulky, sticky precipitates which *adsorb* or *occlude* silt, bacteria and coloring matter. The water is held in large settling basins till most of the dirty precipitate has settled out and it is then run onto the filters. The particles to be removed consist chiefly of the floc remaining after the settling process and, being large, they are readily filtered out by the sand. The filters remain

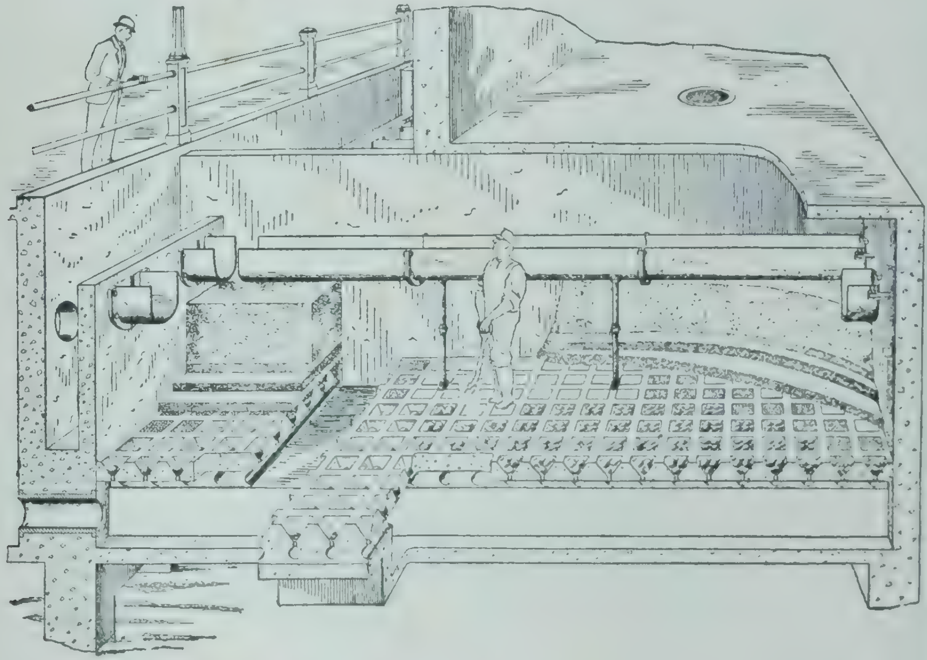

Fig. 282. A rapid sand filter. Diagram showing, in rear corners, the different layers of sand; under foot, the drains for filtered water and the pipes admitting wash water in a reverse direction; at the digger's shoulders, the troughs leading away the overflow from the washing process. (From Horwood, "The Sanitation of Water Supplies," courtesy of Charles C Thomas, Springfield, Illinois, publishers.)

in service for several hours or days, and are then washed by a flow of water and air forced upward through them. Figure 282 shows a rapid sand filter. After filtration the water is treated with chlorine and stored in underground cisterns. Highly polluted waters may be made potable, crystal clear and almost sterile by these devices, and it is due largely to these, as well as to improved methods of sewage disposal, that typhoid fever and cholera remain at a low level or absent in cities with water filtration plants.

Sewage. The purification of sewage in cesspools and city sewage disposal plants is wholly dependent on biological processes in which protozoa, algae and putrefactive bacteria play the chief parts. The processes are, fundamentally, the same as those occurring in natural decay and putrefaction, but are controlled and exploited to the best advantage by the sanitary engineer.[26, 27] Many of the species of soil and intestinal bacteria already mentioned are involved in both processes.

The flora of sewage varies greatly, depending on its age and method of disposal. Fresh city sewage contains dilute feces, along with other city wastes. Consequently it contains the flora of the intestinal tract. This includes many strict anaerobes and facultative anaerobes, mostly saprophytic and heterotrophic. Common types from soil and intestine are *Escherichia coli,* and *E. freundii, Streptococcus faecalis, Clostridium perfringens,* and *Bacteroides vulgatus, Aerobacter, Proteus, Pseudomonas, Cytophaga,* sulfur and iron bacteria, also yeasts, molds and actinomyces.

The saprophytic bacteria actively hydrolyze, ferment and otherwise decompose the organic matter and produce their usual by-products, H_2S, NH_4OH, CO_2, CH_4, lactic, butyric and acetic acids, alcohols, mercaptans, etc. If the process of sewage disposal is conducted in deep tanks, anaerobic decomposition proceeds best, and the facultative bacteria and the clostridia and other strict anaerobes thrive. In aerobic processes (see activated sludge, page 594) aerobic species predominate and odors are less noticeable. As conditions of *p*H, O-R tension, type of organic matter and types of antagonistic bacteria change, the nature of the flora alters.

Sewage Disposal. Of great importance in the prevention of waterborne disease is the proper disposal of sewage. Towns situated on streams below cities which pour raw sewage into the stream are certain to be constantly visited by typhoid fever unless protected by an efficient filter plant, and this has been the experience of many communities in the past. Such towns can sometimes force the city to install a sewage "purification" plant.

The sewage of a city is composed largely of water, and is handled in pumps and tanks much as is drinking water. Sewage disposal plants are operated to accomplish several ends as follows:

1. *Screening;* to remove bulky foreign matter such as bottles, paper, wooden boxes and other extraneous refuse.
2. *Separation* by gravity of all matter in the sewage which will settle to the bottom of tanks of various kinds.
3. *Aeration* or anaerobic biological treatment of the supernatant fluid and possibly final chlorination of the supernatant fluid.
4. *Digestion, and drying of the sludge* or sediment.
5. *Disposal of the sludge.*

Numerous methods have been devised for accomplishing these various ends. The first is largely a matter of mechanics and does not concern us. The second is of interest since it is here that microbial action first comes into play. Various kinds of tanks or "grit chambers" are employed, all designed to allow the solid matter to settle out as much as possible, and in which the sewage is held for some time (twelve to thirty hours) in order to permit anaerobic bacterial decomposition of the organic matter to occur (Fig. 283). Such tanks are called sedimentation or "septic" or digesting tanks. Anaerobic and facultative denitrifying bacteria, proteolytic, fermentative, cellulose-digesting and other types of organisms, protozoan as well as bacteria, all play a role, and most of the organic matter is turned into liquids, gases and soluble materials, greatly reducing the volume of solid matter to be handled and making it much less offensive to the senses and much more stable biochemically. Sedimentation is sometimes hastened by the addition of flocculating agents similar to those used in water purification. The digested sludge is drawn off from the depths of the tanks through pipes.

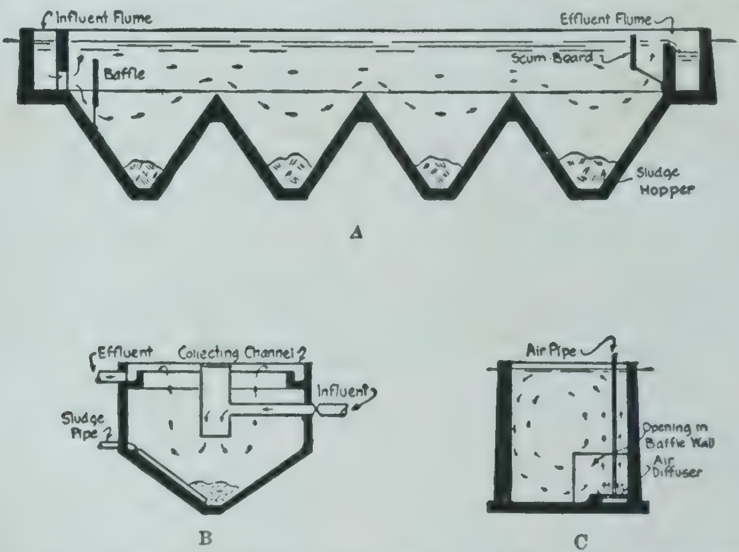

Fig. 283. *A*, Plain sedimentation tank, horizontal flow; *B*, plain sedimentation tank, vertical flow; *C*, activated sludge tank, spiral flow. (Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

"Two-story" Tanks. Some tanks are made in two compartments, an upper and a lower. The upper portion is like a V-shaped trough and serves to introduce fresh sewage. The solid matter settles out through a slot at the bottom of the V into a deep sludge sump as the fluid flows laterally along the trough. The solid matter, after settling to the lower compartment, is held and stored, digestion and decomposition going on there. Gases from the sludge compartment are collected and led to the surface by a funnel-shaped structure in-

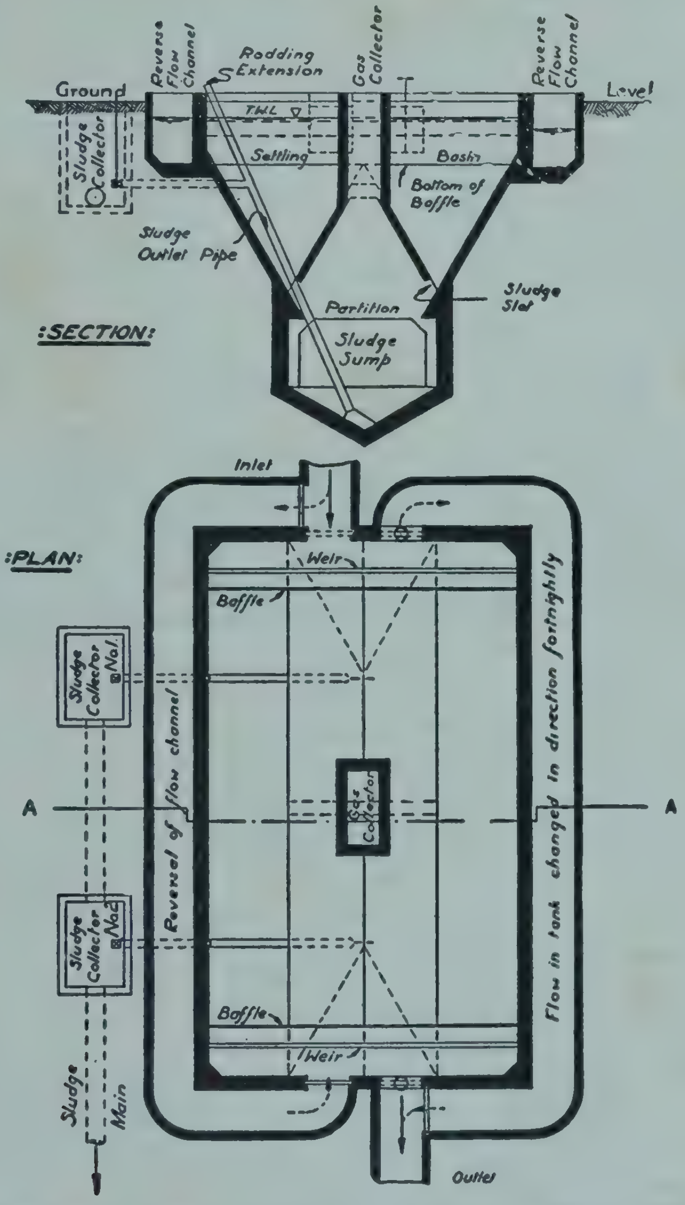
Ground
Reverse Flow Channel
Rodding Extension
Gas Collector
Reverse Flow Channel
Level
T.W.L.
Sludge Collector
Settling
Basin
Bottom of Baffle
Sludge Outlet Pipe
Sludge Slot
Partition
Sludge Sump
:SECTION:
Inlet
:PLAN:
Weir
Baffle
Sludge Collector No.1
Reversal of flow channel
Flow in tank changed in direction fortnightly
A
Gas Collector
A
Sludge Collector No.2
Baffle
Weir
Sludge Main
Outlet

Fig. 284. The Imhoff type of "two-story" tank. (T. P. Francis, "Modern Sewage Treatment," The Contractors' Record, Ltd., publishers.)

verted over the sump. The digested sludge, largely mineral residue, is pumped out from the bottom at intervals. The best known tank of this type is the *Imhoff tank* (Fig. 284).

Aeration and Disposal of Fluid. The fluid part of the sewage after passage through the sedimentation tanks or above the sludge compartment of the Imhoff tank still contains much putrescible organic matter. This fluid is often disposed of, where a porous and dry soil is available, by surface ditches or by a subsurface irrigation system of tile pipes, furnishing excellent fertilizer for farm crops raised on the land. There is no danger of infection as typhoid, dysentery, and cholera organisms are killed either by the antagonistic action of saprophytic bacteria in the settling tanks or soil, or are filtered out by the soil and die.

The fluid may also be allowed to drain through artificial beds of sand, the filtrate being collected by under-drains much as in slow sand filtration of water. This process of purification is similar to those used in filtering drinking water, the sand grains becoming coated with a living film of protozoa and bacteria which feed upon the organic matter of the sewage and upon each other, the result being a much less putrescible liquid. Such filters require aeration daily to allow oxidation processes to occur and are therefore *intermittent* in operation. They must be cleaned at intervals of weeks or months.

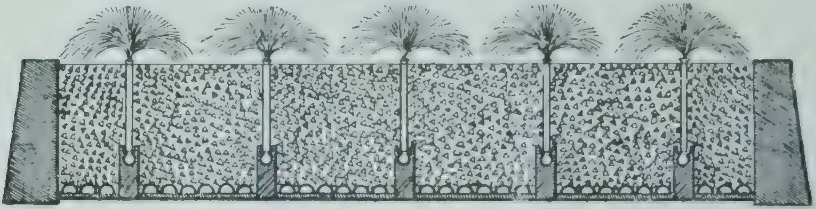

Fig. 285. Typical section of a trickling filter. (Gordon M. Fair, Rosenau, "Preventive Medicine and Hygiene," D. Appleton-Century Co., publishers.)

The beds of sand may be replaced by deep tanks full of coke or broken stone. The tanks are filled with the fluid and allowed to stand so for some hours while the coke or stones, covered with films of living organisms, act like a catalyst, adsorbing and decomposing the organic matter in contact with them. These *contact tanks*, like the intermittent sand filters, must be drained and aerated daily to allow oxidation of the adsorbed matter to occur.

Another type of aerating device, operating on similar principles, is the trickling filter in which the fluid is intermittently sprayed over the stones instead of flooding them (Fig. 285).

Activated Sludge. If air is bubbled actively through sewage in a tank, aerobic conditions are maintained throughout the liquid. Particles of suspended matter flocculate, after a time, into small masses swarming with microscopic life and capable of oxidizing organic matter readily. These masses are called "activated sludge." After a good, active, floc of aerated sludge is formed in a tank, more sewage can be passed through the tank and a great clarification, organic decomposition, oxidation, and decrease in bacterial content is obtained, providing aeration continues and enough activated sludge is intimately mixed with the traveling sewage. The bacterial reduction lies between 90 and 98 per cent. The basic principle of the process is the same as that of aeration and filtration, the sand grains or stones of sewage filters being replaced by the particles suspended in the activated sludge.

Actual operation of an activated sludge plant, with some of the practical problems involved, have been described by Adams.[27a]

Prevention of Water-borne Disease. Disease transmission by water in the absence of elaborate filtration and chlorination systems, as in camping trips, treks, in far-away places, etc., is easily prevented. One needs only to bear in mind that all of the common intestinal pathogens are readily killed by exposure to 70° C. for ten to fifteen minutes and by contact with chlorine and other disinfectants. Knowing this, one can easily guard against these organisms by drinking only boiled water. Water for camp use, provided it is clear and clean, may be treated with chlorine by using one of the methods of field chlorination involving tablets of $CaOCl_2$ available on the market. Our troops became familiar with these tablets during World War II. A recent type uses triglycine hydroperiodide in place of $CaOCl_2$.[28] A teaspoonful of fresh chloride of lime in 25 to 50 gallons of water will also prove an adequate safeguard unless the water is very heavily polluted or dirty. At least an hour must be allowed for the chlorine to act. Ordinary laundry bleaches (Na_2OCl) will also serve. Directions are found on the labels of the bottles.

MARINE BACTERIA

The open sea, as shown by ZoBell,[29, 30] Stanier,[31] and others, may be said to have its own indigenous flora. There are interesting differences in surface flora and the populations of the profound depths. Some Rhodobacteriineae are common in the sea. One group of marine species includes photogenic (light-producing) bacteria.

Photogenic Bacteria. There are several species of photogenic bacteria, some of which occur in the sea and which may be cultivated upon sea-water agar. Some also require peptone. *Pseudomonas phosphorescens,* isolated from luminous marine fish, is one example of a saprophytic photogenic species. At times, photogenic bacteria may grow thickly on the surface of fish or other water animals, causing luminescence (Fig. 286).[32]

Many marine bacteria may be ancestral varieties of terrestrial forms. A number of species of agar-digesting and chitin-digesting bacteria as well as other interesting types have been described in sea water by ZoBell, Stanier, Henrici, van Niel and others. Some resemble members of the soil genera *Flavobacterium, Pseudomonas* and *Cellulomonas;* others are like *Cytophaga* and *Vibrio*. They are adapted to a marine life and are adversely affected by solutions of low salinity. Some are autotrophic.[33, 34, 35, 36] An interesting marine nitrogen cycle is carried on by marine bacteria.[37] Some marine species attack petroleum hydrocarbons (see page 613). The Great Salt Lake of Utah, the Dead Sea, etc., each has its own peculiar flora of halophilic bacteria capable of living in strong salt solution.[35]

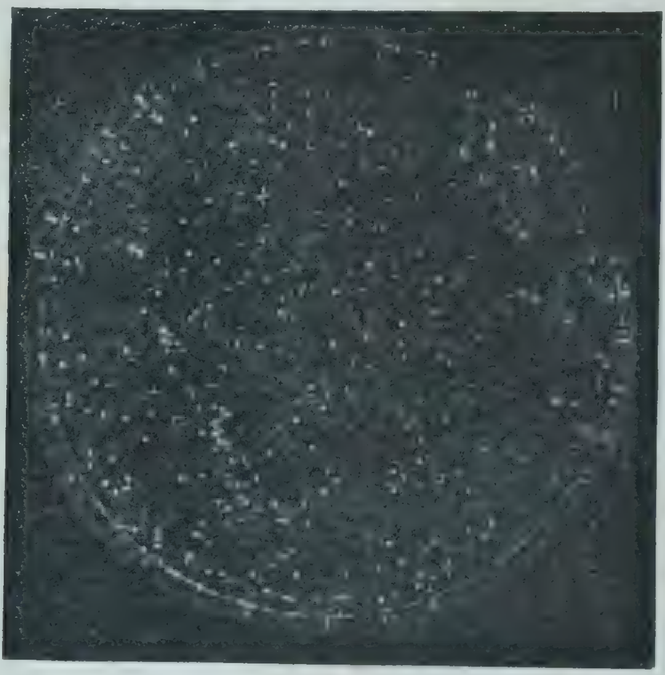

Fig. 286. Colonies of photogenic bacteria. (Lafar.)

Marine Bacteria and Petroleum. Very interesting observations have been made concerning the possible role of marine microorganisms in the formation of petroleum. The various organisms inhabiting deep ocean beds and marine sediments can, as a group, attack almost any sort of organic matter.[38] They are anaerobic and produce low O-R potentials. They metabolize many kinds of organic matter with the production of hydrogen and reduced carbon compounds, like CO and methane. These bacteria can produce more complex hydrocarbons than methane, *e.g.*, carotene. Many such bacteria can produce fatty acids by transformations of fats and oils. It requires only the abstraction of O_2 from fatty acids to form long-chain hydrocarbons. Thus it is clear that anaerobic microorganisms can transform organic substances into petroleum-like matter. Various phases of this subject have been discussed by ZoBell[39] (see also page 613).

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Chapter 35

THE MICROBIOLOGY OF THE SOIL

With the exception of a few highly parasitic organisms like the gonococcus, pneumococcus, etc., most of the organisms described in this book are indigenous to or occur commonly in the soil, so that we cannot cover the entire subject of "soil bacteriology" in this single chapter. However, we may begin by outlining general methods for investigating the bacterial flora of soil and then describe some of the species most important in agriculture and industry. Those of medical importance are described elsewhere. As pointed out by Conn, "The microflora of the soil has been under investigation for at least 70 years; and yet there is at the present time no publication which sums up the existing information in a form sufficiently concise to serve as a guide to any one who is beginning research in soil bacteriology . . ." The same author has summed up the subject very well indeed.[1]

Bacteriological Examination of Soil. Since fertile soil contains anaerobic organisms, strict aerobes, thermophils, autotrophs, heterotrophs, and other varieties having specialized growth requirements, no single method can be given for cultivating or enumerating soil bacteria in general.[1a] Three procedures are available for crude studies yielding approximate results. For exact results the methods of *selective enrichment* (providing a medium or temperature or salinity, etc., which favors one species but not others) or *selective bacteriostasis* (providing a medium which permits growth of a desired species but inhibits others) must be used. Applications of selective methods are described in detail elsewhere, *e.g.,* enteric group.

Plating Methods. The plating method is applicable to the enumeration and isolation of bacteria in any substance, such as soil, water, milk, blood, feces, etc., and suitable modifications are made to meet the cultural requirements of the bacteria likely to be found in the substance examined. In examining soil a small weighed sample (about 1 gm.) is thoroughly shaken up with a measured volume (say 5 ml.) of sterile water. A series of dilutions of the water, which now contains many free bacteria and fine particles of soil to which others adhere, is prepared and 1 ml. of each dilution is placed in a sterile Petri dish. Each dish receives about 15 ml. of melted nutrient agar, cooled to about 45° C. This is thoroughly mixed with the diluted sample by gently tilting and rotating the plate.

After incubation, which may last from twenty-four hours to two weeks, preferably at about 25° C. since many soil bacteria grow well at this temperature, the colonies are counted and the number of organisms per gram of soil computed from the dilution, the weight of the sample, and the volume of wash water.

Dilution Methods. These are special applications of the method of selective cultivation or enrichment. The basic principle is like that underlying the isolation of coliform organisms from water by means of lactose broth or brilliant-green-lactose-bile broth. Dilutions of the soil sample are prepared as for plating but portions of each are placed in special media. For example, a series of dilutions is placed in tubes or plates containing cellulose as the only source of carbon. Only cellulose digesters can grow. If media are used containing no nitrogen and only inorganic substances, only nitrogen-fixing, autotrophic bacteria can grow. From the dilutions in which growth occurs, the approximate numbers of the types for which provision has been made can be stated. Other types are not counted.

Microscopic Examination. By making stained smears of soil and examining them with the microscope we may count various morphological types of bacteria and other microorganisms. The principal inaccuracy in this method is that dead as well as living cells are counted. Another arises from the difficulty in staining some species and still another from errors in differentiating minute soil and other inert particles from bacteria, as well as morphologically similar species of bacteria from each other.

A weighed soil sample is suspended in 5 to 10 times its weight of very dilute gelatin, agar or egg albumen. The viscous substances serve to glue the bacteria to the slide. A measured amount (0.01 ml.) of the suspension is spread over a given area of the slide (1 sq. cm.) dried, and stained with carbol fuchsin, methylene blue or other dye. After washing and drying, the bacteria are counted. For the method of computing the numbers per gram of sample, see the section on direct microscopic examination of milk (page 634).

The methods mentioned above, while inexact, have proven extremely useful and fruitful in soil microbiology. It has been found that bacteria inhabit only the upper layer of soil, seldom occurring in any large numbers below the level of 2 feet from the surface. Good fertile loam may contain anywhere from 100,000 to 500,000,000 per gram. Among these soil bacteria we find every form of bacterial morphology and almost every type of metabolism known to the class of fission fungi.

One of the very important functions of soil microorganisms is cellulose decomposition.

Cellulose Decomposition. In a previous chapter a brief review of the chemical changes involved in this process was given. Here, as part of our discussion of the microbiology of the soil, we may look a little more closely at some of the microorganisms involved. The action of fungi and various actinomyces as cellulose digesters has been known for some time, and cellulose fermentation by anaerobes of the genus *Clostridium* has been described. Considerable interest has centered about aerobic bacteria which digest cellulose. As pointed out by Fuller and Norman,[2] relatively few are *specific* cellulose digesters but are capable of utilizing a variety of carbon sources, of which cellulose is one, and perhaps not the most suitable. Cellulose is often decomposed by the combined and reciprocal actions of a variety of organisms. Among known *specific* bacteria cellulose digesters are *Cellulomonas* (much like *Pseudomonas*), *Cytophaga,* and a large group of curved organisms, including *Cellvibrio* and *Cellfalcicula*. Several species of cellulose-digesting *Pseudomonas, Achromobacter* and *Bacillus* were described by Fuller and Norman.

It is important to note that C. P. cellulose (*e.g.,* filter paper) is utilized with difficulty, especially in the presence of lignin. In wood, other substances besides cellulose aid the growth and action of the bacteria. These other substances include proteins, other carbohydrates, etc. As pointed out by Norman and Bartholomew, ". . . it is not easy to account for the extensive and rapid fermentation of plant cellulose (a mixture) that is accomplished by a mixed flora on the basis of laboratory experiments with pure cultures on pure cellulose.[3]

One of the most interesting aspects of cellulose digestion concerns its symbiotic utilization. While not strictly part of soil microbiology it may be mentioned here. Recent studies have been summarized by Hungate who points out the importance of microorganisms in decomposing cellulose in the rumen of cattle so that it becomes available as food to the animals; a similar relation exists in the guts of termites.[4, 5]

Nitrogen Bacteria. One of the most important groups of soil bacteria are those comprising the families Nitrobacteriaceae, Rhizobiaceae and Azotobacteriaceae, which carry forward the nitrogen cycle. In order to appreciate fully the important functions of these bacteria, it is desirable to consider especially their remarkable metabolic habits.

FAMILY NITROBACTERIACEAE

The nitrogen bacteria are so called because they are concerned in the oxidation of ammonia and nitrites to nitrates in the soil, an important phase in the nitrogen cycle, as we shall see. Nitrates are one of the most expensive forms of plant food. The nitrogen bacteria are autotrophic, and utilize ammonia or nitrites as sources of energy, depending on the species involved. The change from ammonia to nitrates is stepwise and involves two separate groups of organisms, each capable of carrying out only one of the two steps. The process may be outlined as follows:

1. **Ammonium Salts to $NaNO_2$.** Genera *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosocystis* and *Nitrosogloea.*

2. **$NaNO_2$ to $NaNO_3$.** Genera *Nitrobacter,* and *Nitrocystis.*

Probably some of the most primitive species of bacteria known are grouped in the tribe Nitrobacterieae.

The *Nitrosomonas,* et al. are capable of oxidizing the ammonia only to *nitrites,* a process called *nitrosification.* Many higher plants cannot utilize nitrites as their only source of nitrogen, and hence their best development is dependent on the presence of the *Nitrobacter,* et al. which oxidize the nitrites to nitrates (*nitrification*).

These bacteria form a rather heterogeneous group of gram-negative and possibly gram-positive rods and cocci (Figs. 287 and 288). None of them forms spores. The *Nitrosomonas* are motile rods, the *Nitrobacter* are nonmotile rods. They are all aerobic and thrive in warm, moist, slightly alkaline (pH 7–8) soils. They also occur in rivers, streams, etc., and are world-wide in distribution. As judged by test-tube experiments, all live best in the entire absence of organic matter. Lignin appears to interfere with nitrification, as well as with cellulose decomposition.[6]

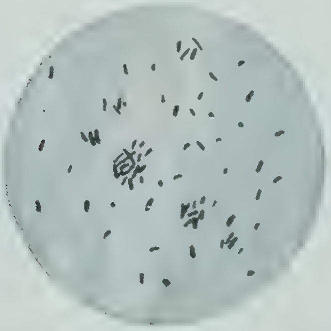
Fig. 287. *Nitrobacter* (× about 900).

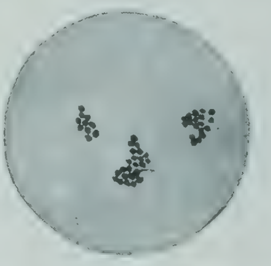
Fig. 288. *Nitrosococcus* (× about 900).

The energy sources of nitrogen bacteria are very different from those of higher forms of life like protozoa, pathogenic bacteria and insects, which utilize only complex organic substances derived from living or dead plants or animals. Consider what is "burned" (NH_4OH and $NaNO_2$) by these autotrophs, and what is the "ash" ($NaNO_3$)! The other elements necessary for the synthesis of the protoplasm of nitrosifying bacteria are obtained from the inorganic salts commonly present in soil or rocks. The *Nitrosomonas* will grow in a solution containing

$(NH_4)_2SO_4$ (the source of energy and nitrogen) . . .	2.0 gm.
K_2HPO_4	1.0 gm.
$MgSO_4$	0.5 gm.
$FeSO_4$	0.4 gm.
NaCl	0.4 gm.
H_2O	1000.0 ml.

A lump of marble or other buffer system is added to prevent acid accumulation.

The nitrifying organisms may be cultivated in solutions such as the preceding by substituting sodium nitrite for the ammonium sulfate.

Thus we find one of the mechanisms by which inert atmospheric nitrogen is made available to complex plants and animals and to man. It may be combined with hydrogen by lightning to form ammonia. It may also be released, in the form of ammonia, from protein by microorganisms which decompose proteins. We see in this mechanism the beginning of a cyclical interchange of nitrogen, which is discussed later as the *nitrogen cycle*.

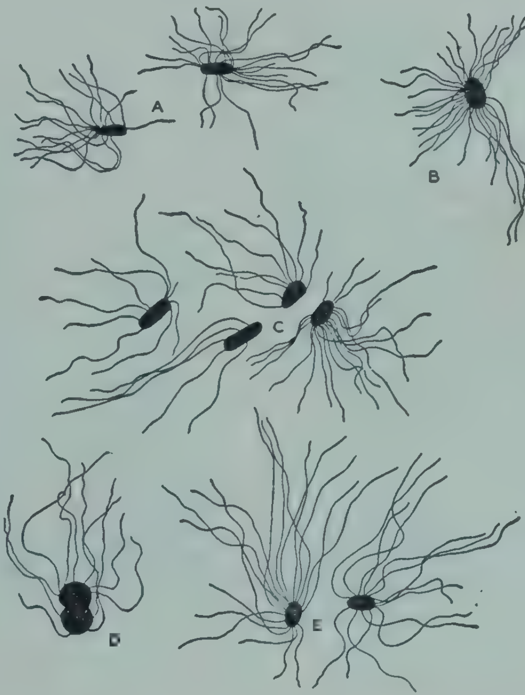

Fig. 289. Drawings of *Azotobacter* flagella using the improved technic. *A*, *Azotobacter chroococcum*, no. 10; *B*, *Azotobacter beijerinickii*, no. 13; *C*, *Azotobacter agile*, no. 8; *D*, *Azotobacter agile*, no. 55; *E*, *Azotobacter indiaum*. (Hofer, A. W., in J. Bact., vol. 48.)

FAMILIES AZOTOBACTERIACEAE AND RHIZOBIACEAE

From the standpoint of the nitrogen cycle and the fertility of soil due to increases in the available nitrogen content by bacterial action, the organisms of the genera *Azotobacter* and *Rhizobium* must be regarded as among the most important. These organisms have the remarkable property of being able to take the inert, uncombined nitrogen of the atmosphere and combine it with other elements to form compounds from which, upon their decomposition, it is liberated in a form available to farm crops either directly or through further bacterial action. This process of synthesizing free nitrogen into compounds is called "nitrogen fixation" and constitutes an important factor in the nitrogen cycle.

Fixation of nitrogen as accomplished by the aerobic *Azotobacter* (and certain species of *Clostridium*) is said to be *nonsymbiotic*, in contrast with the mechanism by which the *Rhizobium* species fix nitrogen in *symbiotic* combination with certain plants. The aerobic, nonsymbiotic fixation of nitrogen will be discussed first.

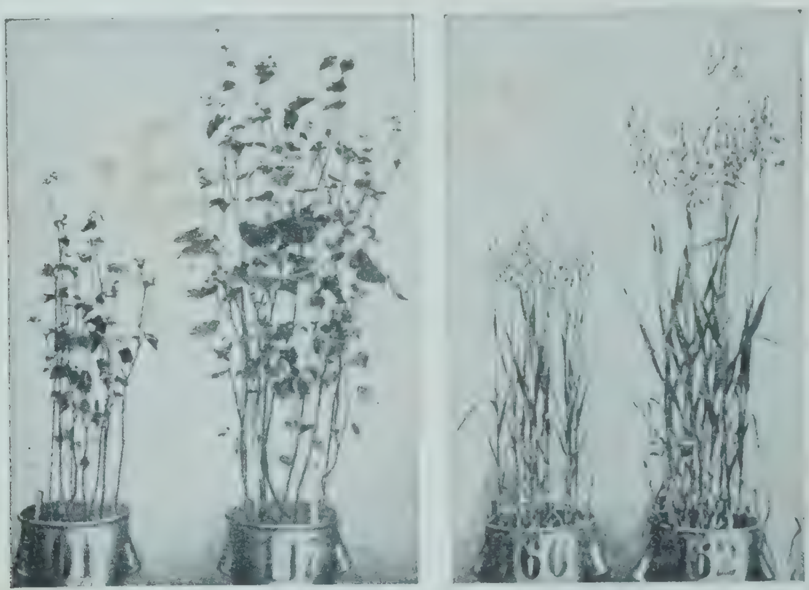

Fig. 290. Buckwheat and oats fertilized with sugar, after A. Koch. Pots 101 and 166 were not treated; 117 and 162 received sugar. (From Löhnis and Fred, "Agricultural Bacteriology," McGraw-Hill Book Co., Inc., publishers.)

Genus Azotobacter. Nonsymbiotic Aerobic Nitrogen Fixation. The *Azotobacter* live in well aerated, slightly alkaline, arable soils and are strict aerobes. They are motile, with numerous peritrichous flagella.[7] They are large, non-sporeforming, usually rod-shaped bacteria, but often grown in oval or yeast-like forms (Fig. 289). They oxidize carbohydrates and other non-nitrogenous organic compounds as a source of carbon and energy and can obtain their nitrogen *directly from the air*. In the soil, the carbohydrates needed for their energy are probably derived ordinarily from the decomposition of the cellulose of higher plants by acid-producing and cellulose-decomposing soil microorganisms. It has been found that carbohydrates added to the soil in the form of molasses, starch wastes and the like act as fertilizer. The fertility results in part from the accumulation of nitrogen in the soil through the growth of the *Azotobacter,* which use the carbohydrates as energy sources (Fig. 290), and combine the nitrogen in their cell-protoplasm, whence it is released on the death of the bacteria. The *Azotobacter* grow readily in such solutions as the following:*

H_2O	1000.0	ml.
Mannitol (source of energy and carbon)	15.0	gm.
K_2HPO_4	0.2	gm.
$MgSO_4 \cdot 7H_2O$	9.2	gm.
$CaCl_2$	0.02	gm.
$FeCl_3$ (10 per cent aq. sol.)	0.05	ml.
Molybdenum salt	Trace	

Adjust to *p*H 7.2. For solid medium, add 15 gm. of agar before adjusting the *p*H.

A solution of this kind will, when inoculated with soil, yield a good growth of *Azotobacter,* and they may be isolated from it on agar plates.

This importance of molybdenum should be pointed out. In the absence of this element in minute amounts nitrogen fixation will not occur. It has been shown also that most species of *Azotobacter* will use ammonia nitrogen in preference to atmospheric nitrogen. Apparently ammonia is the first compound formed from the nitrogen by *Azotobacter.* They can also utilize nitrates, nitrites, urea and asparagine, and will do so to some extent even if atmospheric nitrogen and molybdenum are present. However, under most soil conditions urea is rarely present in a significant amount while ammonia, though constantly being formed by decomposition, is quickly removed by bacterial oxidation or assimilation by higher plants. Nitrates are not often present in a sufficient concentration to decrease fixation of atmospheric nitrogen markedly. Hence, *Azotobacter* ordinarily have little choice in their nitrogen source.[8a]

Nonsymbiotic Anaerobic Nitrogen Fixation. Nitrogen fixation is carried on in swampy soils by at least one species of *Clostridium,* common in boggy, acid soils, where the *Azotobacter* cannot grow. This organism has no relation to the Azotobacteriaceae beyond the fact that it uses atmospheric nitrogen to build its protoplasm and thus fixes atmospheric nitrogen in the soil. It is mentioned here solely because of this. (The genus *Clostridium* is discussed in Chapter 29.) The species in question was described about 1902 by Winogradsky and by him named *Cl. pasteurianum.* It is probably identical with, or very closely related to the type species of the genus, *Cl. butyricum.* Probably several similar organisms of the soil fix nitrogen.

Genus Rhizobium. A very interesting group of nitrogen-fixing bacteria is composed of certain species which, although surviving in the soil, are not always found freely growing there like the *Azotobacter.*

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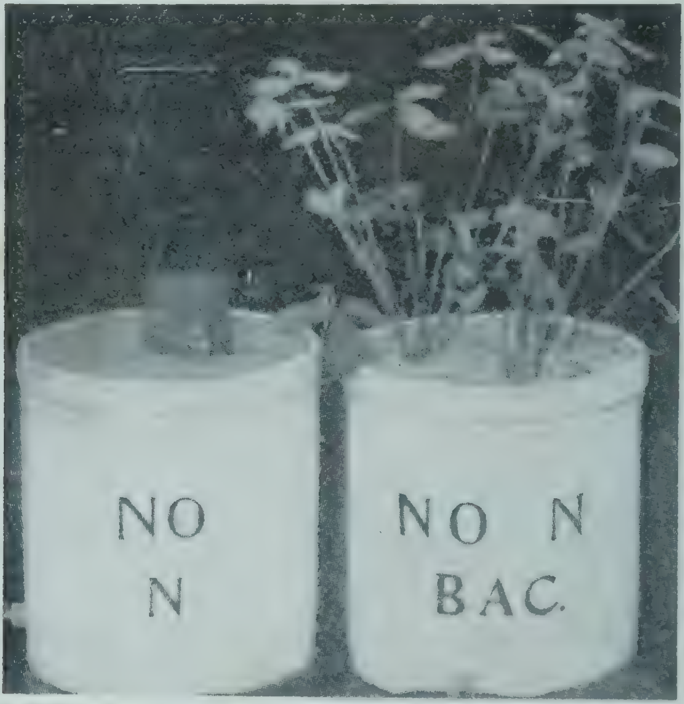
* Note the absence of nitrogen source.

Their most characteristic activity and form is seen when they grow in the tissues of certain plant roots, where they live very well.[9] For this reason they have been given the generic name *Rhizobium* (*Rhizo-* = root).

Six species of the genus *Rhizobium* are described, named chiefly for the type or species of leguminous plants with the roots of which they are characteristically associated. They are *R. leguminosarum*, associated especially with peas and vetches; *R. phaseoli*, infecting bean plants (genus *Phaseolus*); *R. trifoli* common in red, white, crimson and related clovers (genus *Trifolium*); *R. lupini*, especially effective in connection with the lupines (genus *Lupinus*); *R. japonicum* invading the soy bean (*Soja japonica*); and *R. meliloti* which is used in planting alfalfa and sweet clover (genus *Melilotus*).[10, 11] All have in common the property of invading certain root hairs of different leguminous plants (beans, peas, clover, etc.). The invasion induces a small nodular reaction on the root of the plant, somewhat resembling a tubercle (Fig. 292). The bacteria are somewhat specific as to plant host. Thus, *Rhizobium japonicum* produces nodules on the soy bean, whereas *Rhizobium meliloti* will not do so, but the latter bacterium will produce nodules on species related to *Melilotus* such



ig. 291. Red clover; effect of nitrogen-fixing bacteria. No nitrogen in soil of either pot. (After Hopkins, 1904.)

as (alfalfa) (*Medicago sativa*). In the nodules the bacteria live symbiotically with the plant. As will be seen, this is of very great benefit to agriculture, as the nodules aid in gathering nitrogen. Strains of any given species vary greatly in respect to nodule formation, some being "good," others "poor." Good strains form relatively small numbers of large, pinkish nodules near the main roots. These root nodules, or pictures of them, have doubtless been seen by every student of elementary biology and they can be found on clover, peas, beans and other legumes in any garden.

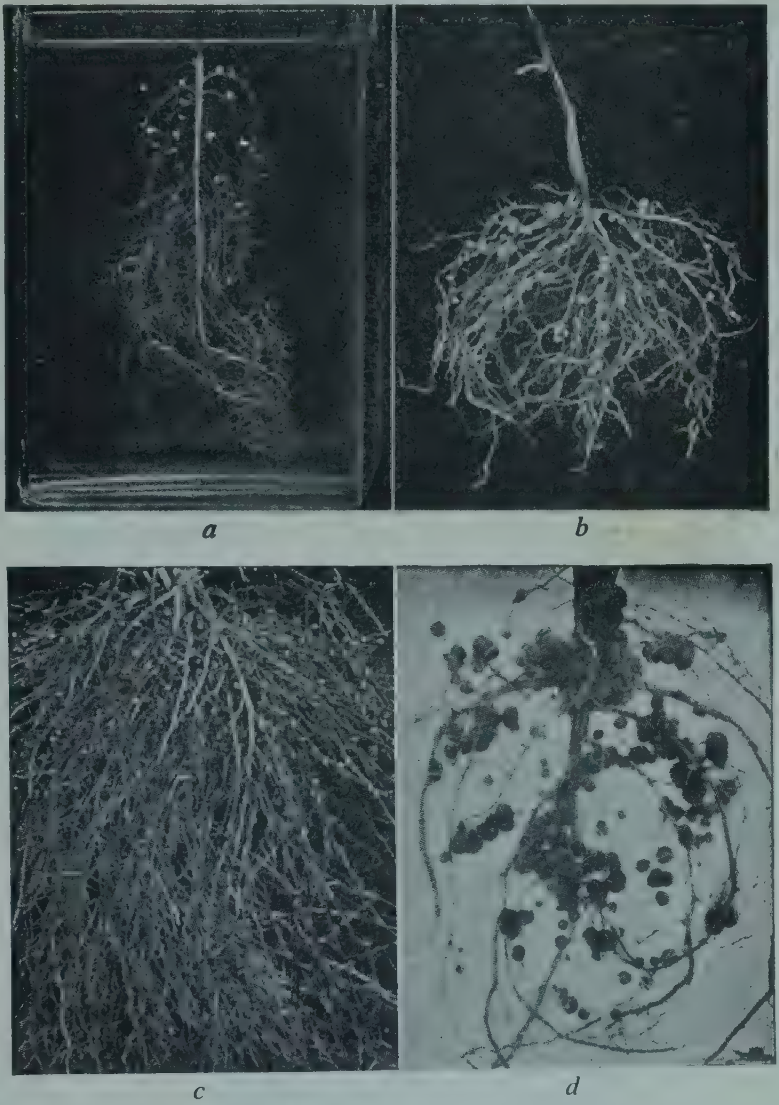


Fig. 292. Roots (with nodules) of (*a*) young alfalfa, (*b*) young pea, (*c*) mature red clover, (*d*) mature soy bean. (From Löhnis and Fred, "Agricultural Bacteriology," McGraw-Hill Co., Inc., publishers.)

The bacteria themselves are of peculiar form and are said to pass through various complicated life stages. However, it has been shown by Lewis that these "life stages" are merely periodic appearances due to the accumulation of reserve food granules, fat vacuoles and the like (Fig. 293). The bacteria may be observed in smears made from crushed root nodules, as Y-, L- and T-shaped, stellate or star-shaped, and ordinary rod-shaped cells (Fig. 294). They are motile when young. They are gram-negative, non-sporeforming, strict aerobes

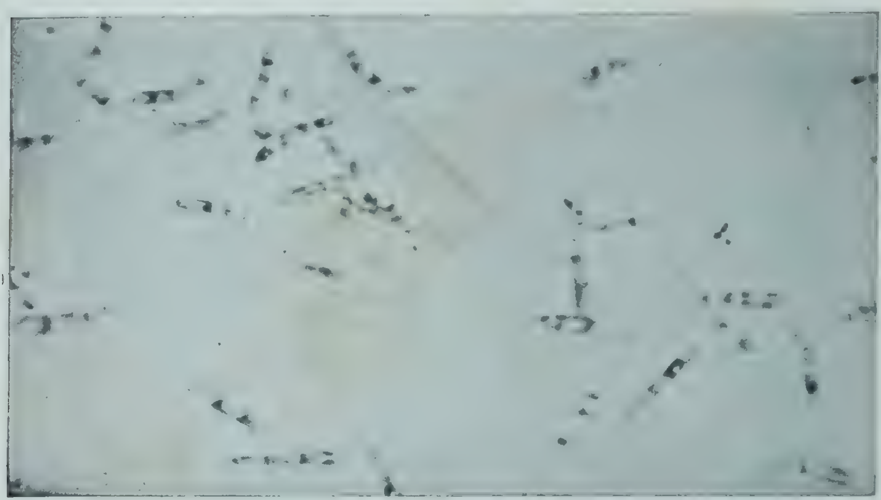


Fig. 293. Cells of *Rhizobium meliloti* showing fat vacuoles and other portions of the cell. (Lewis, I. M., J. Bact., vol. 35.)

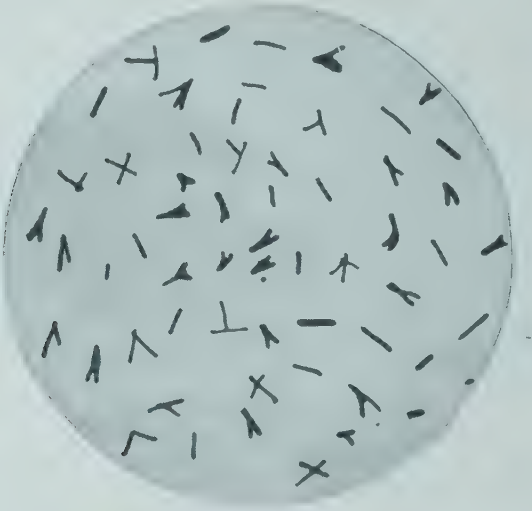


Fig. 294. Various forms of *Rhizobium* (× 900).

and may be cultivated on such materials as pea-broth gelatin containing asparagine, or such broth as the following:*

H_2O	1000.0 ml.
Glucose	20.0 gm.
KH_2PO_4	1.0 gm.
$(NH_4)_2SO_4$	0.5 gm.
$MgSO_4 \cdot 7H_2O$	.01 gm.
NaCl	Trace
$FeSO_4$	Trace
$MnSO_4$	Trace
$CaCl_2$	Trace

Agar may be added to stiffen the medium. Adjust the *p*H to about 7.2.

They utilize various carbohydrates, forming characteristic gummy or mucilaginous substances in cultures. These gummy coverings are of importance in helping them invade plant roots, forming a sort of hollow thread through which the cells migrate into the tissues.[11, 12]

Symbiotic Nitrogen Fixation. The rhizobia utilize carbohydrates and other substances found in the juices of the host plant and, like the *Azotobacter,* take their nitrogen directly from the air. This is synthesized by them to compounds from which it is yielded to the plant in which they live. The plants, when young, can utilize fixed nitrogen (nitrates) in the soil, but if this is absent and the proper species of *Rhizobium* for nodule formation are not present in the soil, they cease growth and die for lack of nitrogen. For this reason it is customary to inoculate virgin soils, or soils not known to support growth of legumes, with the proper species of *Rhizobium* preparatory to planting such crops as alfalfa or soy beans for the first time. State and Federal departments of agriculture often make suitable cultures available to farmers. They are also available on the market under various trade names. The cultures are usually mixed with the seed before planting.

The root nodules and tissues of legumes contain large amounts of combined nitrogen prepared by the bacteria and thus it is that the cultivation and plowing under of crops like clover, vetch or soy beans enriches the soil with "fixed" nitrogen as well as with the other valuable organic constituents of the whole plants. Inoculation of swampy acid soils is money wasted, as *Rhizobium* will survive and grow only in fertile, dry, aerated and neutral, or nearly neutral, soils. Crops of nonlegumes grown in association with legumes (as vetch and rye or clover and corn) have for centuries been known to be superior. The legumes apparently excrete fixed nitrogen into the soil.

The Nitrogen Cycle. The nitrifying, and nitrogen-fixing bacteria are of interest and value because they introduce into the soil in available form an element (nitrogen) which is absolutely essential to life and which is chemically so inert that it never combines spontaneously or readily with other elements, but only at the expenditure of great quantities of energy such as is found in lightning flashes or in huge electrical furnaces or other expensive apparatus. The bacteria produce combined nitrogen very cheaply, efficiently, and unostentatiously.

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* Note that in this medium a source of combined nitrogen is included since the bacteria are not growing symbiotically when cultivated in vitro.

However, were all nitrogen continuously to be used by living things and combined in the form of protoplasm and to remain so, inextricably bound up as protein, then the agricultural use of manures, animal carcasses, fish fertilizers, etc., would be of no avail. Were dead animals not to decay, manure not to rot, and dead fish to remain dead fish, the only forms of nitrogen available would be ammonia from lightning and free atmospheric nitrogen and all plants and animals would have to await the slow activities of the nitrifying or nitrogen-fixing bacteria, or be limited to the use of the rare atmospheric ammonia or to ammonium salts, which are not very favorable, in order to obtain properly combined nitrogen. Such, however, is not the case.

Organic Decomposition. As soon as protoplasm ceases to live, and as soon as any organic matter returns to the soil, it begins to undergo spontaneous oxidative changes and also the biological decomposition process of *decay*, which is aerobic decomposition, or *putrefaction* and *fermentation*, which are anaerobic decomposition of proteins and carbohydrates, respectively.

The microorganisms causing decomposition are, for the most part, saprophytes and heterotrophs but are frequently capable of autotrophic existence. There are numerous aerobic and anaerobic forms: molds, yeasts, cocci, bacilli, actinomycetes, and spirochetes. Each attacks one or more of the complex substances which compose dead plants and animals—cellulose, bone, hair, proteins, fats, carbohydrates—hydrolyzing or otherwise decomposing them and utilizing them as sources of energy or carbon or oxygen or nitrogen or some other element. One species may utilize and decompose the metabolic products formed by another, and so on, until the complex organic materials become so simple, chemically, that they are again available as food for plants (Fig. 295).[13]

The microorganisms of the soil thus live in a constant state of mutual assistance or *symbiosis*, interdependence, and competition and antagonism. So active are microorganisms in attacking organic matter in the soil and utilizing the various elements of which it is composed, especially nitrogen, that the plowing under of manure or straw or other organic fertilizer just before planting may prove deleterious to the crop. The microorganisms multiply in the fertilizer to such an extent as to offer serious temporary competition to the crop plants for the available nitrogen and other elements. Some bacterial products (acids, etc.) are also deleterious to crops and to other bacteria. Liming of the soil is then desirable. Later, of course, the microorganisms die and disintegrate, liberating their nitrogen.

With regard to the nitrogen of the decomposing organic matter, which alone concerns us for the moment, it is released from protoplasm and from animal and plant wastes chiefly through decompositions involving *ammonification, deamination* and *denitrification.*

Ammonification and Denitrification. In a previous chapter an outline was given of the biological processes by which protein is first hydrolyzed to amino acids and these broken down to other, simpler compounds when they are utilized as energy sources or in cell synthesis by microorganisms. Ammonia resulting from deamination is commonly produced in bacterial protein decomposition. The release of ammonia from the decomposition of organic nitrogen compounds is called ammonification. Note that the nitrogen is in its most reduced form.

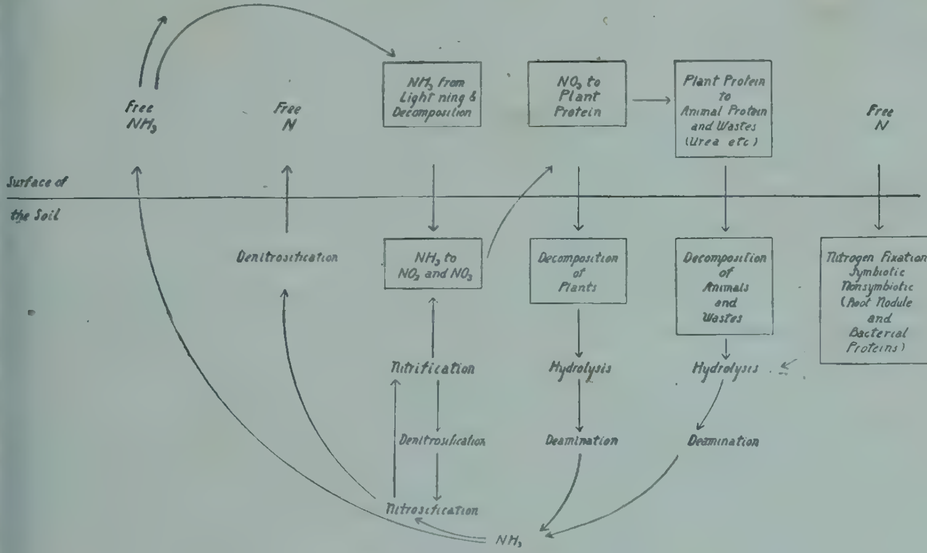
Free NH_3
Free N
NH_3 from Light ning & Decomposition
NO_3 to Plant Protein
Plant Protein to Animal Protein and Wastes (Urea etc)
Free N
Surface of the Soil
Denitrosification
NH_3 to NO_2 and NO_3
Decomposition of Plants
Decomposition of Animals and Wastes
Nitrogen Fixation Symbiotic Nonsymbiotic (Root Nodule and Bacterial Proteins)
Nitrification
Hydrolysis
Hydrolysis
Denitrosification
Deamination
Deamination
Nitrosification
NH_3

Fig. 295. The nitrogen cycle.

The fixed nitrogen represented by ammonia might escape, in part, from the soil, in the same manner that much is lost to the atmosphere from manure and compost heaps. It would then be lost to the living cycle were it not for its combination as ammonium salts and for the bacteria (*Nitrosomonas,* etc.) which oxidize it to nitrites. The nitrites would be in great part useless to plants were it not for other bacteria (*Nitrobacter*) which oxidize them to nitrates.

The reverse of nitrification is referred to as *denitrification.* In denitrification, nitrates are used by various microorganisms of the soil to act as hydrogen acceptors and so are reduced. The extent of the reduction process depends on the species involved and the availability of free oxygen. Many aerobes and anaerobes reduce nitrates to nitrites (denitrification). This step in the process is commonplace, nitrates apparently yielding one molecule of oxygen with little difficulty. Some familiar species of facultative bacteria reduce nitrites to nitrogen but this process is not so common a process as nitrate reduction. The nitrogen may also be further reduced to ammonia by these or still other species. Some bacteria are incapable of anaerobic growth unless nitrates or similar compounds are present to furnish an acceptor of hydrogen.

The decomposition of proteins and of nitrates with liberation of nitrites, nitrogen or ammonia accounts in part for the lack of fertility of constantly wet soils containing organic and other matter furnishing food for ammonifying or anaerobic denitrifying species. Some of these species might be *Thiobacillus denitrificans* or various clostridia.

Thus we see that nitrogen, like sulfur, is in a constant state of alteration between an oxidized state and a reduced state as long as it remains in the living system. Its introduction from the inert atmospheric state into the cycle of life is in great part dependent on bacteria.

OTHER PRIMITIVE SOIL BACTERIA

Certain bacteria of the soil are of considerable interest because of their curious metabolism, especially their sources of energy. One group of these bacteria is called the genus *Hydrogenomonas*. These oxidize hydrogen as a source of energy and use part of this energy in cell synthesis.

The reactions are probably as follows:

$$\left.\begin{array}{l}1.\ 2H_2 + O_2 \longrightarrow 2H_2O \\ 2.\ 2H_2 + CO_2 \longrightarrow (CH_2O) + H_2O\end{array}\right\} \textit{Hydrogenomonas pantotropha}\text{, etc.}$$

These bacteria inhabit chiefly swampy places where microbial decompositions of organic matter are releasing considerable amounts of free hydrogen. They are facultative autotrophs, motile rods, and form heavy capsules around themselves. Some species are said to form spores. They may be isolated, after some experience, on organic media like milk-agar or nutrient gelatin. Hydrogen, of course, is an efficient fuel, and its oxidation, resulting in the formation of water, yields abundant energy, part of which may be used in the chemosynthetic reduction of carbon dioxide as shown in reaction 2 above. But surely it is a strange and unsubstantial food.

In addition to these curious bacteria of the soil, there is a group which has a strange and potentially exceedingly important relationship to international politics and industry. These are the organisms involved in the formation and destruction of petroleum.

Microbiology of Petroleum. No final conclusions as to the mode of origin of petroleum may be reached on the basis of present knowledge. However, it is generally held that it originated from living organisms. Crude petroleum contains, in addition to various hydrocarbons (paraffine, kerosene, etc.) which are carbon-hydrogen compounds, compounds of nitrogen, sulfur, phosphorus and elements in proportions and relations suggestive of their derivation from organic matter. Studies of the subject make it pretty clear that: (*a*) the temperature of petroleum formation was within a range compatible with bacterial life (30 to 80° C.); (*b*) pressures were well within the limit of microbial viability; (*c*) petroleum was formed in or near its present locations which, at the time, were probably sea bottom.

As shown by ZoBell, et al., the sea bottom, as well as oil reservoir fluids and oil from deep oil wells, contain large numbers of microorganisms, including bacteria, which grow under the environmental conditions prevailing in oil deposits: temperatures of from 0° to 85° C.; pressures up to 150,000 pounds per square inch; water of varying degrees of salinity; completely anaerobic. These organisms are capable of metabolizing virtually any sort of organic matter, including phenol, rubber, and other compounds generally thought of as refractory to microbial decomposition, or as poisonous to microorganisms. Under the anaerobic conditions of marine ooze very low O-R potentials (—0.5 volt) are developed, and reduced compounds are formed as a result of microbial metabolism. Methane (CH_4), which results commonly from anaerobic bacterial metabolism at very low O-R potentials, is a hydrocarbon common in petroleum. The formation of C_2H_6 (ethane) and C_3H_8 (propane) and long-chain compounds under similar conditions is easily conceivable and has actual experimental support. For example, in experiments with various organisms, including sulfate reducers which are common in deep sea ooze, normal paraffines (C_2OH_{42} to $C_{25}H_{52}$) and crude oil waxes were produced, apparently in part from caproic acid, and under conditions simulating those thought to surround the formation of petroleum. Reactions like the following are postulated:

$$C_6H_{12}O_6 + 7H_2S \longrightarrow C_6H_{14} + 6H_2O + 7S.$$

An important role of microorganisms in petroleum formations may be liberation of oil now diffusely scattered in hardly perceptible amounts in various rocks. In calcareous materials, microbial formation of acids would dissolve the rocks and permit confluence of oil globules into the hollow spaces formed by action of the acid on the rock. Gases formed could force the oil through crevices in rocks, such as sand and shale, into larger collections.

Many plants synthesize hydrocarbons. Huge deposits of these, such as the deposits which formed coal, when decomposed by certain microorganisms, could liberate large amounts of the hydrocarbons found in petroleum.

Destruction of Petroleum. On the other hand, there is a large group of organisms which attack and destroy petroleum hydrocarbons. Some of these are illy classified primitive bacteria of the soil which have curious metabolic properties and are included in the genera, formerly called *Carboxydomonas* and *Methanomonas*. These oxidize simple carbon compounds as energy sources, as follows:

1. $CO + \frac{1}{2}O_2 \longrightarrow CO_2$. *Carboxydomonas oligocarbophila*
2. $CH_4 + 2O_2 \longrightarrow CO_2 + 2H_2O$. *Methanomonas methanica*

Like *Hydrogenomonas,* they are small, motile rods growing in marshes and swamps where hydrogen, carbon monoxide and methane are bubbling up owing to the anaerobic decomposition of the organic matter, chiefly cellulose, in the depths of the bog. They are autotrophs and are aerobic. Two interesting species of the genus *Methanomonas* are M. *aliphatica* and M. *aliphaticoliquefaciens*. These oxidize the higher homologues of methane, such as certain of the petroleum oils and paraffin.

Many other microorganisms can attack and decompose hydrocarbons in gasoline, etc., and may be of considerable importance in the petroleum industry as causes of spoilage. Among these, as shown by Stone, Fenske, White,[14] and ZoBell,[15, 16] and others, are *Pseudomonas* and *Achromobacter* species, also *Alcaligenes, Mycobacterium, Aspergillus, Monilia, Sarcina,* etc.[17]

The use of such bacteria, and of marine forms having similar properties, to find hidden sources of petroleum is of interest. Culture mixtures, complete in all respects except carbon source, are placed in flasks and inoculated with an appropriate species of organism able to utilize petroleum vapors as carbon source. On being lowered into suspected oil-bearing strata and left for some days, growth will occur if hydrocarbon (petroleum) vapors are present. Thus the bacteria in the hands of skillful bacteriologists serve as a sensitive reagent for petroleum sources.[18]

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Chapter 36

THE MICROBIOLOGY OF THE AIR

No particular species of microorganisms inhabit the air. Microorganisms of the air, like those in fresh surface water, are merely those found in soil, manure, etc., which have become attached to dust particles and are blown about by the wind.[1] Microorganisms are more numerous in air in dry weather than just after a rain, because, while rain contributes greatly to the numbers of microorganisms in surface waters, it washes them out of the air and "lays" the microbe-laden dust.

Obviously only species resistant to desiccation and exposure to sunlight can persist long in the air. Microorganisms commonly found in air are spore-bearing bacilli like *Bacillus subtilis* and *Clostridium perfringens,* micrococci like *Sarcina lutea* and *Micrococcus luteus,* nonpathogenic corynebacteria (diphtheroids), chromogenic and nonchromogenic non-sporeforming rod like the coliform group, *Serratia marcescens* and its allies the *Chromobacterium* and *Flavobacterium,* and the spores of yeasts and molds. Actinomycetales are also often found. In fact, almost any of the species of microorganisms already discussed in this book may at times be found in the atmosphere. Much depends on the location where air samples are examined, weather, air currents, population, etc.

In the dust and air of theaters, schools, hospital wards or the rooms of persons suffering from infectious bacterial diseases such organisms as tubercle bacilli, streptococci and pneumococci have been demonstrated, as well as other bacteria such as diphtheroids and staphylococci common in the normal mouth and throat. These are dispersed through the air by coughing, sneezing, talking and laughing (Fig. 155). Viruses may also be transmitted by air.[2, 3, 4, 5] The kinds of organisms present in air obviously depend on what sources of bacteria are being stirred up in the vicinity and on the direction from which air currents are flowing as well as their velocity, and the temperature and humidity. If the atmosphere is undisturbed, all dust will settle and the air becomes sterile (see Tyndall's experiment on spontaneous generation, page 17).

The bacteria of the upper air have been collected by means of aeroplanes or other aerial devices. For the collection of bacteria in the upper layers of the atmosphere, special apparatus must be used which excludes any possibility of contamination of the culture material, or other collection device, by dust from the airship or its occupants if such be used.[6, 7] Microorganisms are found in air even over the polar regions and far out at sea.[7a]

Collection and Enumeration of Aerial Microorganisms. There are numerous devices for collecting microorganisms in the air. Most of them are designed for use indoors in the study of atmospheric pollution as related to air-borne diseases. For the latter purpose, there are three general types of devices: impingement devices, bubbling devices, and atomizing devices.

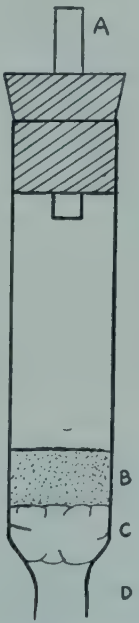


Fig. 296. Tube for collecting dust from the air for bacteriological analysis. Air enters at *A*, deposits its dust on the sand (*B*), which is supported by a cotton plug (*C*), and leaves at *D*.

Impingement Devices. These depend on the use of Petri plates or other vessels containing nutrient agar of composition appropriate to the sorts of microorganisms to be collected. In the simplest procedure, the plates are set about on the floor of rooms and allowed to remain open for specified periods of time. Dust, droplets of saliva from coughing and sneezing persons, etc., fall on the agar plates, which are then incubated. The colonies are later counted and different types of organisms may be separately enumerated if desired. This method is more qualitative than quantitative, but gives a rough idea of the number and types of bacteria present in a given atmosphere. Like similar methods used in counting bacteria in milk and water, it is incomplete because some of the bacteria present will not grow under the cultural

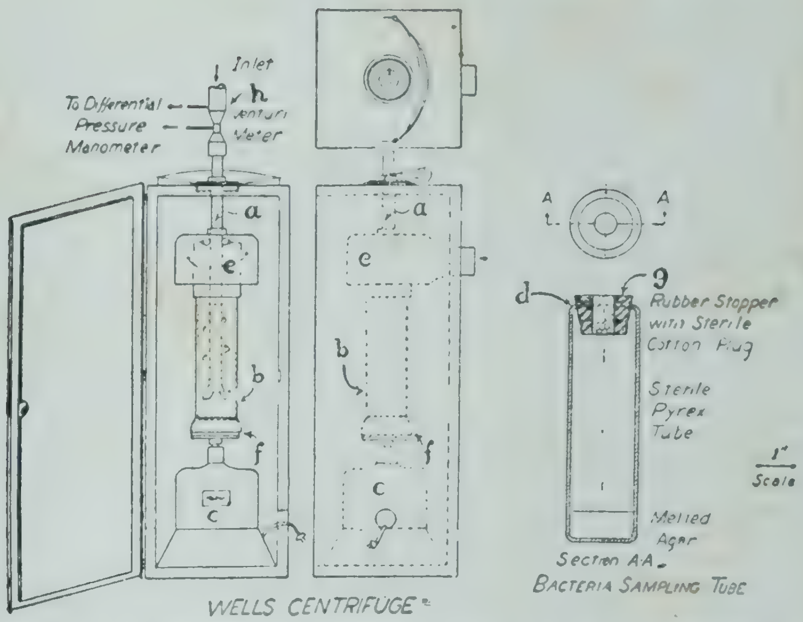

Inlet
h
To Differential Pressure Manometer
Venturi Meter
a
e
b
f
c
A
A
d
g
Rubber Stopper with Sterile Cotton Plug
Sterile Pyrex Tube
1"
Scale
Melted Agar
Section A-A
Bacteria Sampling Tube
WELLS CENTRIFUGE

Fig. 297. Air centrifuge and bacterial sampling tube. *a*, Inlet tube; *b*, metal sleeve, carrying the sampling tube; *c*, motor; *d*, turned in rim of the sampling tube; *e*, outlet; *f*, rotating support for the sampling tube; *h*, inlet with Venturi meter. (Reproduced from Wells, Am. J. Public Health, vol. 23.)

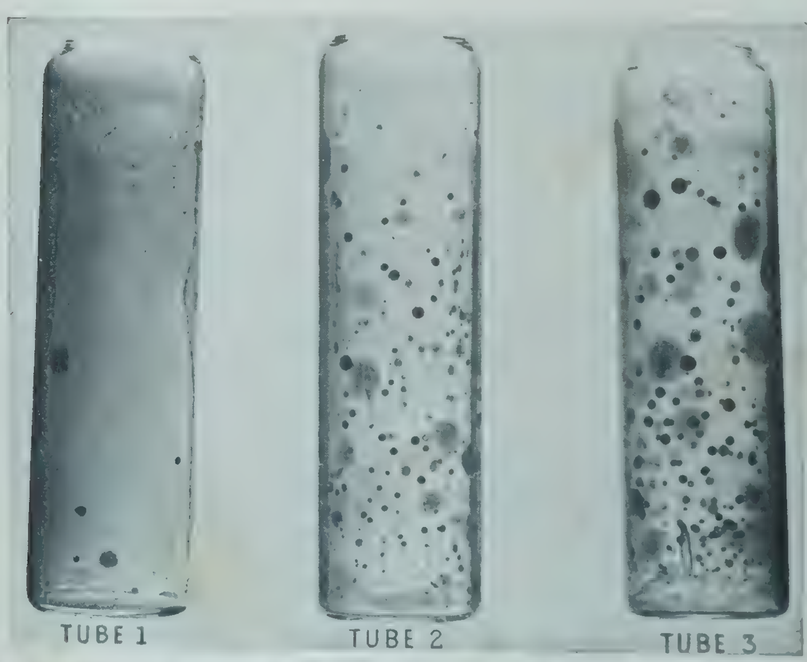

TUBE 1
TUBE 2
TUBE 3

Fig. 298. Bacterial colonies in air-centrifuge cylinders, showing different densities of bacteria in various parts of a hospital. Samples of air (1) premature ward, (2) and (3) children's wards. (Courtesy of Dr. C. F. McKhann.)

conditions provided. Special media may be used for the enumeration of certain kinds of bacteria, for example, blood agar for hemolytic species.

An inherent property of impingement devices is that, as in counting bacteria in milk and water by plating methods, clumps of organisms often form only single colonies, so that the total number of colonies found is smaller than the actual number of organisms present.

The Air Centrifuge. This is a device for causing a whirling current of air to pass through a rapidly rotating glass cylinder lined with a film of nutrient agar medium appropriate to the bacteria to be obtained from the air and dust. The infected dust particles cling to the sticky agar film and the cylinder is eventually closed at each end and incubated. Colonies may then be counted and fished for identification. With a knowledge of the volume of air passed through the cylinder, the density of contamination of the air with each kind of bacterium may be estimated (Figs. 297, 298).[8]

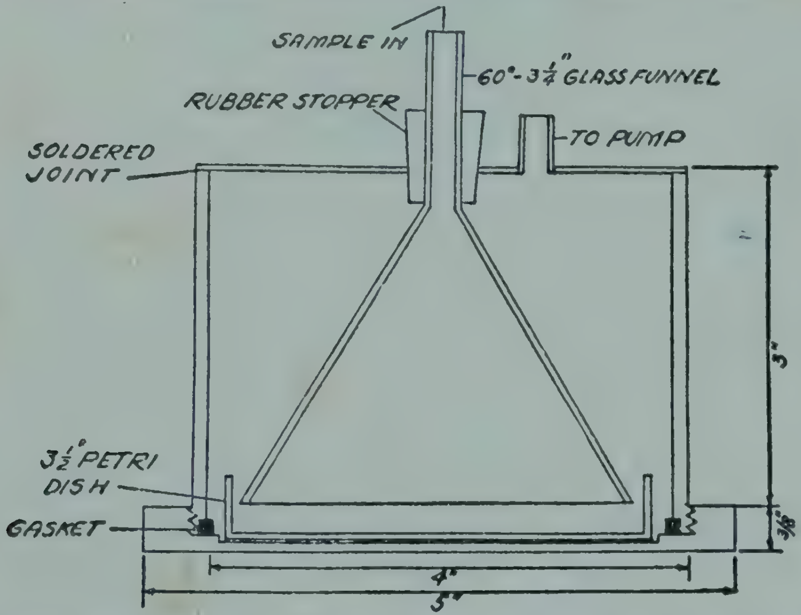


Fig. 299. Funnel device, with funnel, standard petri dish, metal cover with outlet, and metal base plate. The metal cover screws onto the base plate. (du Buy, H. G., Hollaender, A., and Lackey, M. D., in Supplement No. 184, U. S. Public Health Service.)

The Funnel Device. There are several devices in which an agar plate or other agar surface is placed beneath an inverted funnel. Air is passed through the device in such a way that dust and microorganisms impinge on, and cling to, the agar surface. After passage of the desired volume of air through the device, the agar is incubated and colonies counted. A simple form of funnel device is seen in Figure 299. In a modification of the funnel device, the agar surface is given a high voltage positive or negative electrostatic charge, while the funnel holds an equal and opposite charge. The air-borne microorganisms impinge on either surface because they also have positive or negative electrical charges.[9]

The Slit Device. This consists of a vacuum chamber containing an agar plate mounted on a slowly rotating disc. About 1 mm. above the

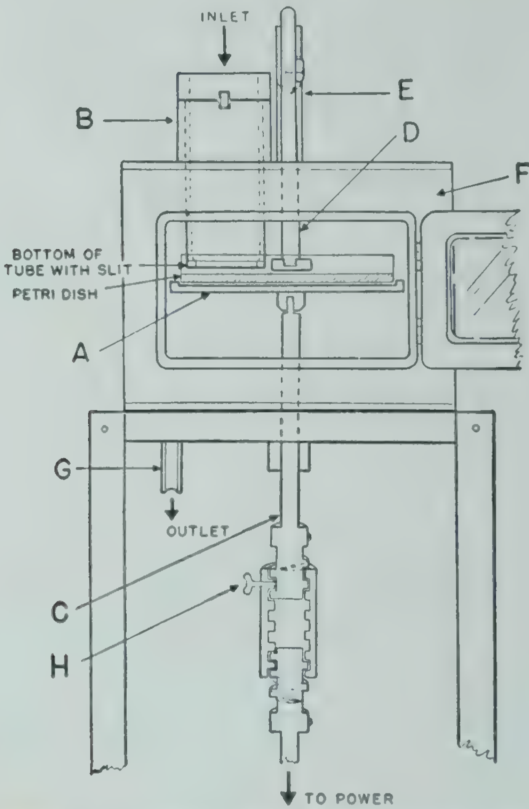
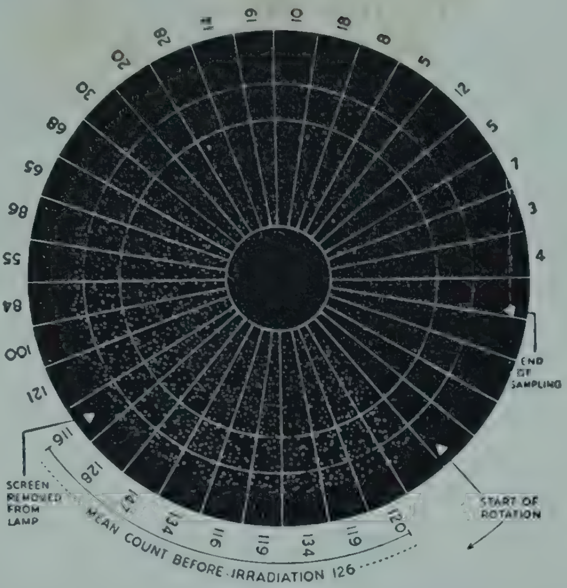


Fig. 300. Slit device. *A*, Rotating table; *B*, tubular air inlet with slit; *C*, rotating axis; *D*, indicator rod; *E*, sleeve, guiding indicator rod; *F*, metal box with door and rubber gasket; *G*, outlet; *H*, turnbuckle with set screw. (Adopted from Bourdollin, et al., J. Hyg., vol. 41, in Supplement No. 184, U. S. Public Health Service.)

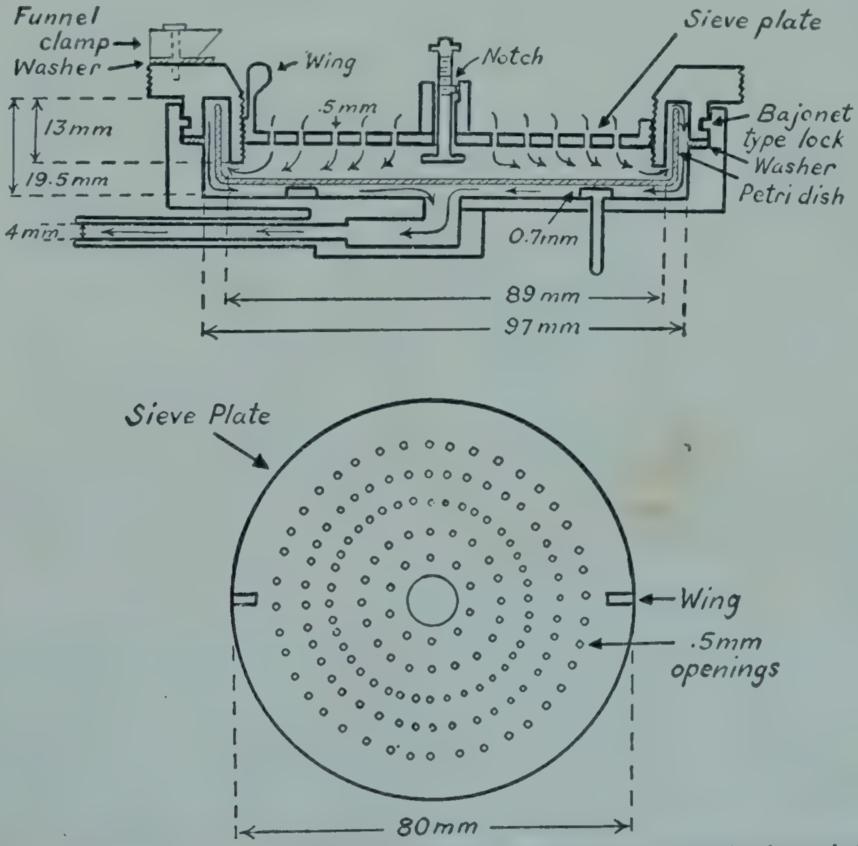
surface of the agar, and extending from the periphery to the center of the plate is a flat, narrow, air duct (a slit) through which air is drawn from the exterior at a considerable speed, so that aerial dust and organisms impinge on the agar. As the plate slowly rotates, variations in density of air-borne organisms are reflected in different numbers of colonies in various sectors of the plate exposed beneath the slit (see Figures 300 and 301).[9]

The Sieve Device. In principle this is similar to the slit device.



10
18
8
5
12
5
1
3
4
END OF SAMPLING
START OF ROTATION
120
119
134
119
116
134
147
128
116
121
100
84
55
86
65
68
30
20
28
11
19
SCREEN REMOVED FROM LAMP
MEAN COUNT BEFORE IRRADIATION 126

Fig. 301. Typical record from a plate taken in a slit sampler. (Courtesy of C. F. Casell & Co., London, England.)



Funnel clamp
Washer
Wing
.5mm
Notch
Sieve plate
Bajonet type lock
Washer
Petri dish
13mm
19.5mm
4mm
0.7mm
89mm
97mm
Sieve Plate
Wing
.5mm openings
80mm

Fig. 302. Sieve device, with box and cover, containing a standard petri dish. Sieve plate with wings is drawn separately. (duBuy, H. G., Hollaender, A., and Lackey, M. D., Supplement No. 184, U. S. Public Health Service.)

In place of the slit a metal disc perforated with many tiny holes is mounted close over the agar surface. Air passes through each perforation, the aerial particles impinging on the agar surface just beneath it (see Fig. 302).[9]

Bubbling Devices. In these air is caused to pass down a tube to the bottom of a vessel containing a volume (10 to 50 ml.) of nutrient broth, through which the air bubbles, releasing its microorganisms as it passes up. After passage of a measured volume of air, the vessel is shaken, and plate counts are made of the organisms in the fluid, in the same manner as milk or water plate counts. The vessel may contain beads to help break up the bubbles and clumps of bacteria, or the opening of the air tube beneath the surface of the liquid may be subdivided into many tiny perforations for the same purpose. A drop or two of olive oil may be floated on the surface to prevent foaming.[9] A useful apparatus of this type is seen in Figure 303.

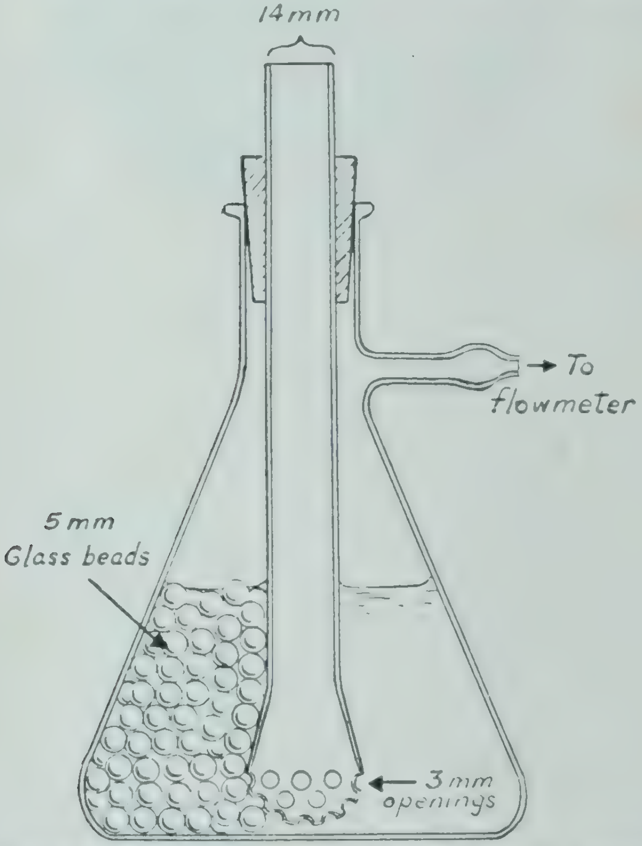


Fig. 303. Bead-bubbler device, consisting of a 250-ml. suction flask, glass beaks 5 mm. in diameter, and a glass bubbler, kept in place by a rubber stopper. (Reproduced from Wheeler, et al., Science, vol. 94.)

Atomizing Devices. These depend on the principle of atomization to produce a layer of liquid around each bacterial particle. The bacteria-laden mist is then adsorbed in a chamber containing a sterile mixture of broth and olive oil. The atomizing and collecting chambers are rinsed with the collecting fluid and plate cultures are made of measured quantities of the mixture. Certain corrections for loss by evaporation are applied. A flow meter measures the quantity of air sampled.

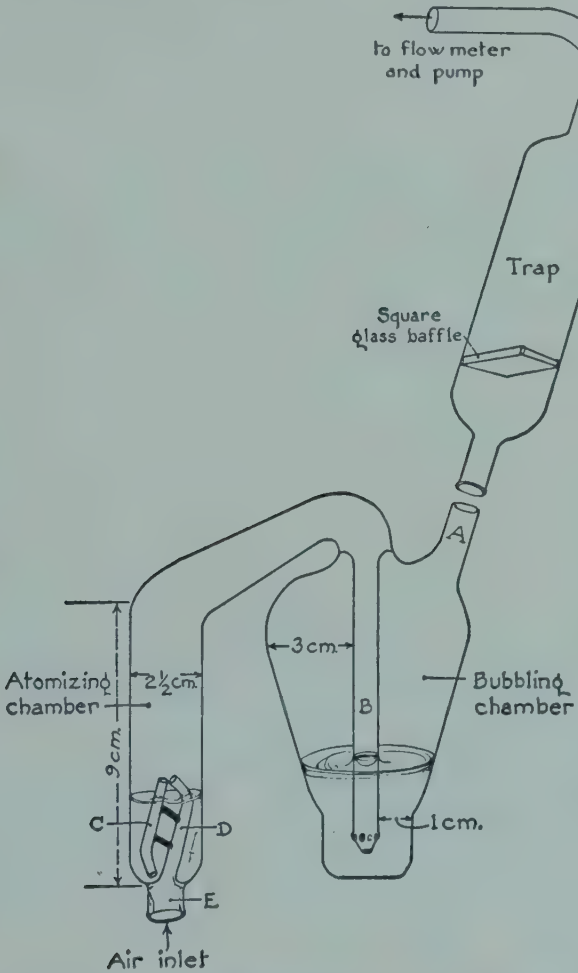

to flow meter and pump
Trap
Square glass baffle
A
3 cm.
2½ cm.
Atomizing chamber
Bubbling chamber
B
9 cm.
C
D
E
1 cm.
Air inlet

Fig. 304. Device for collecting bacteria from air. (Moulton, Puck and Lemon—Science, vol. 97.)

One type of collecting tube is shown in Figure 304. Air enters at E, passes through D and draws a mist of fluid through C. The droplets fall back into the fluid in the first chamber or are collected in the bubbling chamber as they pass through B. The baffle and trap prevent any loss of fluid.

The bubbling and atomizing devices yield higher counts per volume of air than the impingement devices because they tend to break apart clumps of microorganisms, dust particles, etc. These and other devices, as well as comparative studies with each, are discussed in more detail in articles cited in the bibliography.[9]

Dust, Droplets and Droplet Nuclei. Bacteria do not mount into the air unaided. Conidia of fungi, and even fragments of mycelium and possibly yeast cells may be blown about by themselves, but bacteria are usually attached to particles of dust unless they happen to have come from the respiratory tract, when they are usually found coated with saliva or mucus. Even the latter soon become attached to dust particles.

Dust usually arises from furnace ash, soil, fabrics, soot, etc. The particles are usually relatively large (10–100 μ) and tend to settle rapidly. Dust particles tend to be trapped in the upper respiratory tract and removed by mucus and saliva flow, sneezing, etc. Only the smallest reach the lungs as a rule.

Droplets, as referred to the respiratory tract, consist of particles of saliva and mucus and may contain hundreds of bacteria. Usually they are of the magnitude of 100 μ, and, like dust, tend to settle rapidly in quiet air and be trapped in the upper respiratory passages. They are usually formed by sneezing and coughing.

Droplet nuclei are the solid residua of droplets after drying. Small droplets may, in a warm, dry atmosphere, dry before they reach the floor and become droplet nuclei. These are small (2–10 μ) and light and may float about for many minutes or even hours. Being very small, they tend to pass the traps of the upper respiratory tract and enter the lungs.[10]

The size, weight, moisture content and opacity of air-borne particles are of great importance in considerations of methods of sampling air for bacteriological examination, and of methods for disinfecting air. For example, impingement methods will tend to catch larger and heavier particles, but not very small and light ones. Ultraviolet rays, sunlight, etc., may reach and kill organisms in droplets or nuclei but opaque dust will protect the microorganisms in them. Humid atmospheres (about 50 per cent) have been shown to be more lethal than very dry or very moist atmospheres (20 per cent or 80 per cent).[10a]

Air Pollution. Two important questions in studies of pollution of indoor atmospheres by human beings are: (1) what organism shall serve as an index of pollution by saliva and respiratory mucus? (compare use of *E. coli* as an index of sewage pollution); and (2) what shall be used as a standard medium? (compare standard media for milk and water examination). Final answers to these questions have not been formulated. As a test organism *Streptococcus salivarius,* a streptococcus of the alpha hemolytic type always (?) found in the normal (?) mouth, has been proposed. Objections are that it may occur elsewhere, and that it is a fragile organism which soon disappears from the air. In other words, does its presence truly indicate salivary pollution; does its absence prove the reverse?

Various media have been proposed. Much depends on the results desired. For general purposes the media used in milk or water examination will serve. For recovery of *S. salivarius* a reliable one appears to be that of Schneiter et al. However, the field has not been fully explored and new methods will be developed from time to time.[11]

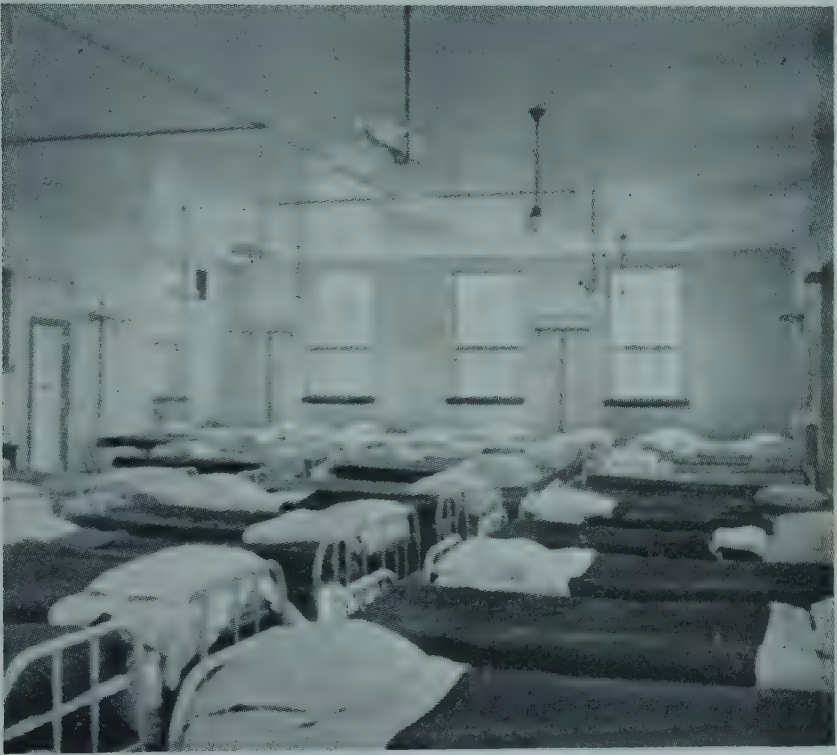


Fig. 305. Ultraviolet light installations in typical sleeping quarters. (Schneiter, R., Hollaender, A., Caminita, B. H., Kolb, R. W., Fraser, H. F., DuBuy, H. G., Neal, P. A., and Rosenblum, H. B., in Am. J. Hyg., vol. 40.)

Control of Air-Borne Infection. It is obvious that under many conditions disinfection or sterilization of the air is highly desirable. In recent years the problem has been given increasing attention, and three general methods are now available for the control of microorganisms in the air. This does not take into consideration such procedures as passing air through mechanical filters, spray washing devices, etc., which are not generally applicable.

Radiation. A method widely discussed is the irradiation of the air with *ultraviolet light* which, as pointed out in a previous chapter, is lethal to bacteria. A radiation of wave length 2537 A° is generally used as this is sufficiently bactericidal and at the same time not excessively irritating. Application of this is being made in schools, factors, storage warehouses, and hospitals. Ultraviolet-producing electric lamps are attached to the walls (Fig. 305), overhead (Fig. 306) or at strategic points between hospital beds or in corridors, etc. Deflectors are used to prevent direct exposure to the rays, which can cause serious "sunburn," and to protect the eyes. A difficulty arises from the necessity that the microorganisms must rise well above the heads of the people in the room in order to come within the range of action of the lethal rays. Only the lighter particles do this. Also, dust is little affected, and only places directly exposed are disinfected.

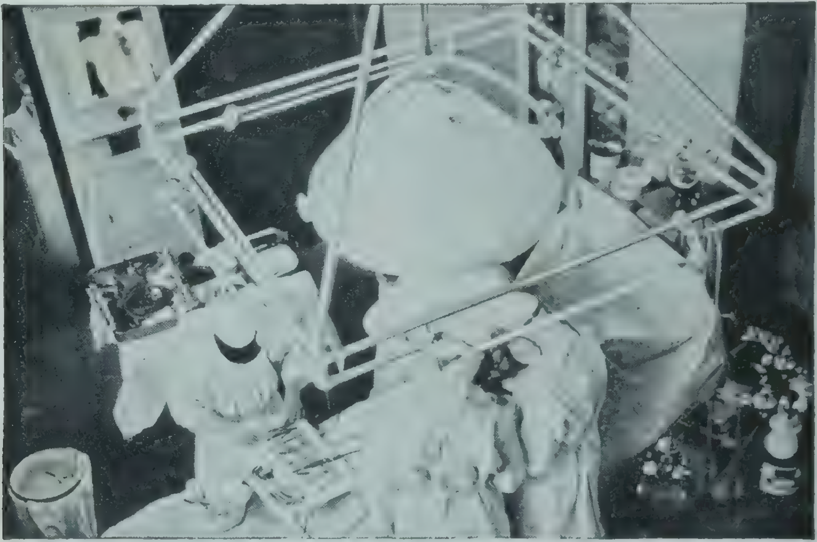


Fig. 306. Maintenance of asepsis in surgery by means of bactericidal radiation from above. The large dome is for illumination. Ultraviolet light is given off by mercury vapor arcs in the rectangular frame of tubes. Note the eyeshades on the surgeons. (Courtesy of Westinghouse Electric & Manufacturing Co.)

Bactericidal Vapors. Many substances are lethal to bacteria in the vapor phase, and several have been used for disinfection of air in years past. Probably the most effective are propylene glycol and triethylene glycol. These are odorless, tasteless, nonirritating, nontoxic and not explosive or corrosive. They are highly effective in killing bacteria in the air although relatively ineffective *in vitro*. That it is the *vapor* molecules, and *not droplets* of fine mist of these substances that are the effective disinfecting agent has been conclusively shown by Puck.[12] As little as 0.5 mg. of propylene glycol in the vapor phase per liter of air can virtually sterilize heavily contaminated air in 15 seconds.

Triethylene glycol is almost 100 times as effective. Mists are much less effective.

Difficulties in the use of these vapors are chiefly of an engineering nature. Discussion of these problems is found in the literature cited.[13, 14] Air conditioning appears inevitable to their effective use. The temperature and humidity of the air are important factors. If the air is cold and dry, or excessively humid, the effectiveness of aerosols is reduced. Relative humidities of around 40 per cent at about 76° C. are favorable.

Dust Control. In a previous chapter (20) it has been pointed out that dust control is important in preventing disease transmission and the fact is reemphasized here. Methods were described in the earlier discussion. An excellent review of this phase of the subject has been published by Loosli.[14a]

Effectiveness of Methods. Each of the methods mentioned above has been shown beyond doubt to be effective in reducing the *number of aerial bacteria,* both in experimentally contaminated atmospheres under various controlled conditions, and in such places as barracks, hospital wards, etc. Combinations of dust control by means of oil, and either vapors or irradiation, are even more effective. When it comes to reduction of *disease,* however, data are less conclusive. The status of the problem is well summarized in two admirable reviews to which the reader desiring details is referred. A quotation from each is given here: "Conclusive evidence is not available at present that the air-borne mode of transmission of infection is predominant for any particular disease." [or that] "traditional methods of controlling contact infection [*e.g.,* as by handshaking, kissing, eating utensils, etc.], can be ignored or relaxed." . . . "The available evidence strongly indicates that methods of air disinfection (ventilation, ultraviolet irradiation, and glycol vapors) are useful adjuvants to aseptic techniques in the reduction or elimination of air-born infections in operating rooms and in contagious disease and pediatric wards."[15]

"Admittedly, both ultraviolet light and glycols have their merits and demerits. Present knowledge is too limited to make any definite claims or predictions concerning their ability to reduce air-borne infection."[16] These views are well borne out by more recent experimental data.[17]

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Chapter 37

THE MICROBIOLOGY OF MILK

In a preceding chapter we have seen the importance in milk of streptococci like *Str. lactis* and of beta hemolytic streptococci of the Lancefield groups A and B. Here we may discuss the presence of other species of bacteria in milk, and indicate their sanitary and other signficance. The microbiology of manufactured dairy products, like cheese, is discussed in the chapter on Industrial Microbiology.

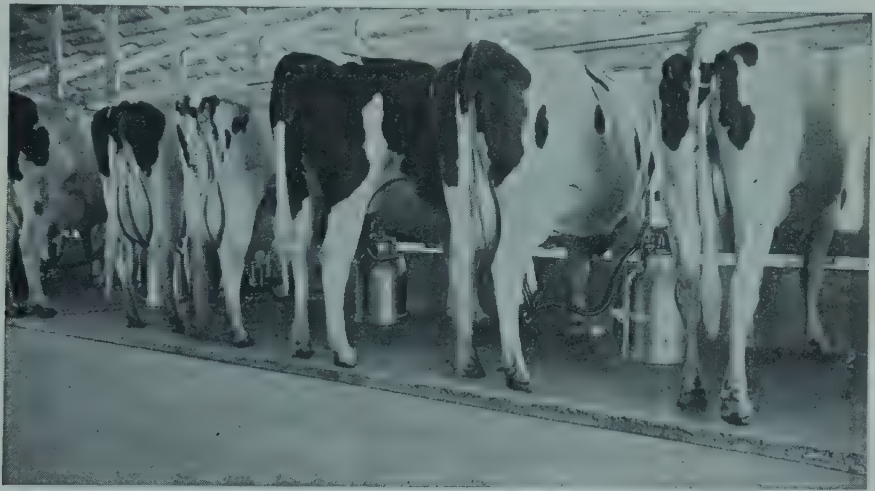

Fig. 307. Sanitary milk production. Note the cleanliness of the cows, the absence of dung and straw, the good lighting and ventilation and the milking machines. (Courtesy of the DeLaval Separator Company.)

Milk secreted into the udder of healthy cows is sterile. It has a *p*H of around 6.8. Some bacteria are able to grow up into the milk duct of the teat so that the first milk drawn (this should be discarded) usually contains numbers of them. These may include staphylococci, micrococci and saprophytic types such as *Bacillus, Serratia,* etc., from soil. Except in cases where extra precautions are taken at the time of milking (see Figs. 307, 308 and 309), the milk receives its largest numbers of organisms from the pail and other dairy utensils, from soil and dust in the air, from the flanks, tail and udder of the cow, and from the hands of milkers (Fig. 310).

Types, Numbers and Significance of Bacteria Normally in Milk. Ordinary market milk always contains strains of *Streptococcus lactis,* enterococci, *Escherichia, Aerobacter,* and other nonpathogenic Enterobacteriaceae, as well as *Staphylococcus, Micrococcus,* Actinomycetales, *Lactobacillus,* sporeformers, yeasts, molds, etc., derived from the intestinal tract of the cows, from barnyard dust, utensils or hands. The presence of these usually *nonpathogenic* bacteria in milk is a serious matter only when they cause the milk to sour, putrefy, or develop undesirable flavors or conditions like "blue milk" or "red milk" or "ropiness" (see page 573), or when, because of their numbers, they show the milk to be stale or dirty. The development of many of these organisms may be prevented by pasteurization, which consists in heating the milk to 145° F. (62° C.) for thirty minutes and immediately refrigerating.

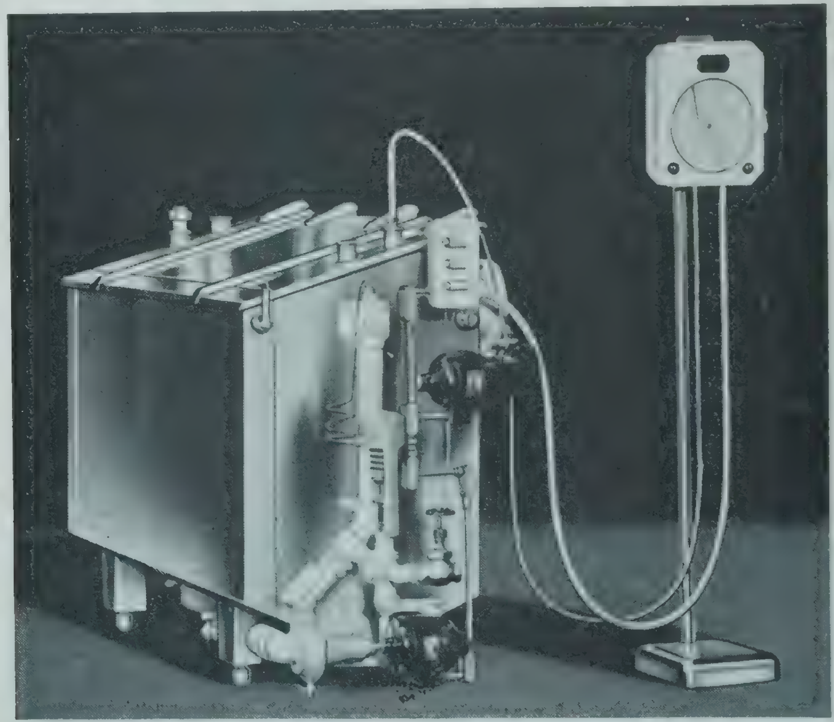

Fig. 308. Modern type of "cc" type of spray pasteurizer showing the tank and external apparatus for controlling the flow of milk and the hot water stream which heats the milk in the vat. An important part is the recording thermometer and electrical temperature control apparatus. The motors operate milk and water pumps and agitate a paddle in the vat to keep the milk circulating. (Courtesy Cherry-Burrell Corp., Chicago, Illinois.)

Since milk is an excellent medium for bacterial growth, the number of bacteria in it will increase steadily the longer it stands, especially if not pasteurized or refrigerated. Even if it is refrigerated, so that mesophilic growth is retarded, psychrophilic species may grow rapidly. Unpasteurized milk "sours" (coagulation or "curds" occurs at *p*H 4.8), due principally to the rapid growth of the lactose-fermenting *Str. lactis,* heat-resistant lactobacilli and coliform organisms. Pasteurized milk usually does not sour so promptly because many of these organisms, especially coliforms, are destroyed by the heat. It may putrefy instead of souring because of the growth of proteolytic spore-formers (*e.g., B. subtilis,* etc.), and other heat-resistant species which resist pasteurization and which may not ferment lactose but attack casein. Yeasts and molds may predominate later on, since many of these are aciduric and can develop in soured milk.

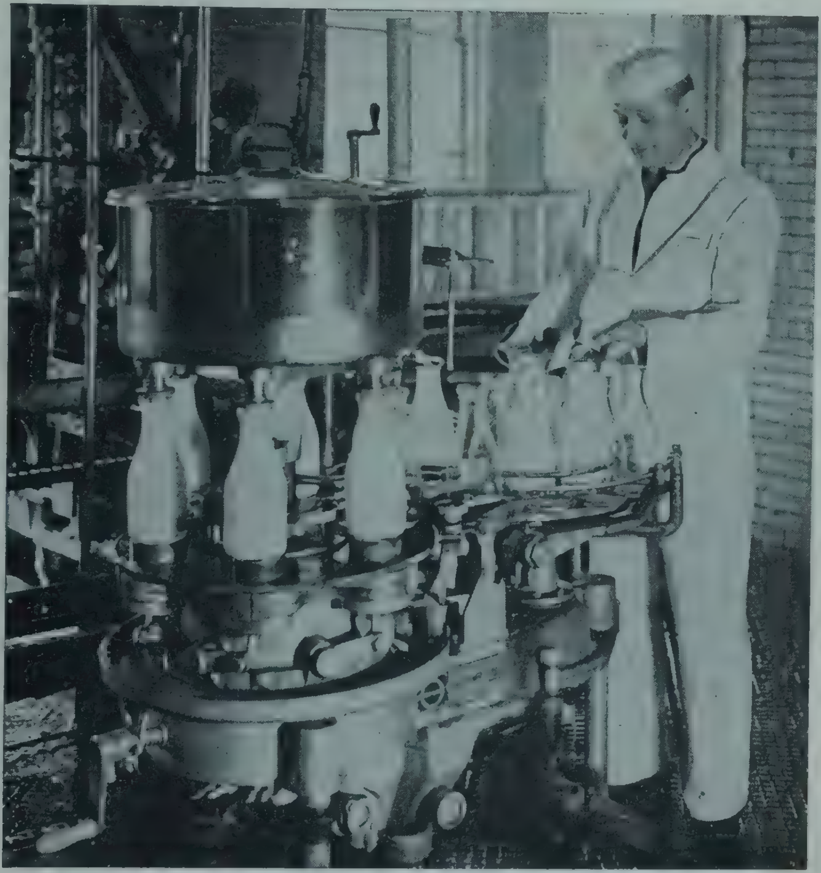

Fig. 309. Sanitary bottling of milk. Compare with conditions shown in Fig. 310. (Courtesy of Cherry-Burrell Corporation, Chicago.)

Stale or dirty milk, therefore, may contain millions of bacteria per ml. The very best quality, fresh, certified "baby milk" may contain from a few hundred to about ten thousand, while good, general market milk may contain ten to fifty thousand organisms per ml.

Obviously, in order to produce milk that is clean, highly sanitary and has good "keeping" qualities, it is necessary to (*a*) have clean cows which are inspected periodically by competent veterinarians to be sure that they are free from disease; (*b*) have all utensils absolutely clean and as nearly sterile as possible (plenty of very hot water or "steam hose"). The use of paper milk bottles has much to recommend it. Studies by Tanner and others have shown the sanitary advantages[1]; (*c*) insist on cleanliness of barns, clothing and person of milkers; (*d*) have all workers in and around the dairy examined medically each month or so.[2]

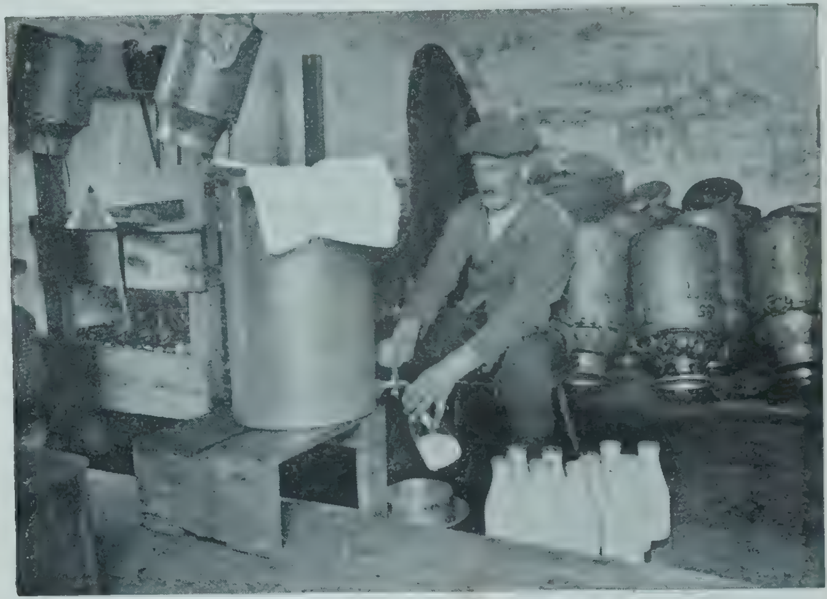

Fig. 310. Unclean bottling of unpasteurized milk. (Photo by Lewis Hine. Courtesy of Cleanliness Institute.)

Significance of Coliform Organisms in Milk. Coliform organisms are always present in market milk before pasteurization. However, these organisms are killed if the process is properly carried out. It is possible to determine this by making counts to determine if any coliforms have survived the heating. Samples of the processed milk are diluted and cultured by methods very similar to those used for determining coliforms in water (see page 581). If considerable numbers of coliforms (more than about 5 per ml.) are found it is evident that the milk has not been properly pasteurized or that it has been excessively contaminated by unclean conditions afterward.

Another method of detecting improper pasteurization or handling of milk is the phosphatase test.

The Phosphatase Test.* This test is based on the power of the heat-sensitive enzyme, phosphatase, normally present in fresh milk, to liberate phenol from phosphoric-phenyl esters. When milk is heated, this enzyme becomes progressively inactivated. When heated at 143° F. for thirty minutes (pasteurization), 96 per cent of the enzyme is destroyed, and heating above 145° F. for thirty minutes insures complete inactivation. When the milk has been underheated (in respect to either temperature or time) or when there is an admixture of raw milk afterward, the enzyme will be present in larger amounts than when the milk was properly processed. Quantitative determination reveals the degree of faulty pasteurization or subsequent contamination with raw milk.

This test will detect 0.5 per cent raw milk mixed with pasteurized milk, or 1 degree below standard temperature, or five minutes' underheating during pasteurization. A color greater than that in a standard of boiled milk with 0.01 mg. phenol per ml. indicates improper handling of milk.

Significance of Pasteurization. Over all ranges of temperature and times, *Mycobacterium tuberculosis* (the most resistant of the non-sporeforming infectious pathogens found in milk) is destroyed more quickly than phosphatase, so that a heat treatment adequate to inactivate the enzyme likewise kills this organism and *all other common pathogenic bacteria.* A sample of milk that does not have more phosphatase present than the standard allows can be regarded as safely pasteurized and free from subsequent contamination with raw milk.

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* The Phosphatase Test. *Rapid Method for Use in the Field.* (Scharer, 1938).[3]

Reagents

A. 2,6-Dibromo-quinone-chloroimide (referred to below as BQC) dissolved in 95% ethyl alcohol and kept in tightly stoppered dark bottle. Remains stable for several days. Use with dropper delivering 50 drops per ml.

B. Buffered substrate contains disodium phenyl phosphate, sodium borate buffer, and magnesium. Dissolve in distilled water. Remove traces of free phenol by treatment with BQC and extraction with butyl alcohol. Substrate unstable and should be stored under refrigeration.

(A and B may be purchased ready prepared, or see ref. above.)

Method

1. Add 5 ml. buffered substrate to 0.5 ml. milk sample. Shake briefly.
2. Incubate 10 minutes in water bath at 37° C.
3. Remove and add 6 drops BQC solution. Shake well.
4. After 5 minutes, compare color with opaque standards (see ref. above).

Properly pasteurized milk will be gray or brown.

Properly pasteurized cream will be gray or white.

Raw milk or cream will be an intense blue. Intensity of color is proportional to degree of underpasteurization or amount of added raw milk.

Certain bacteria, as *Lactobacillus enzymothermophilus*,* discovered and identified by Buck and further reported by Kaplan, will give falsely positive results in properly pasteurized milk because they produce a heat-stable phosphatase.[4, 5]

Counting the Bacteria in Milk. In order to have some measure of the conditions under which milk has been produced and handled and to have a legal control over its sanitary quality, health departments and dealers have set up various standards by which to judge milk.[2, 6] Important among these standards is the number of bacteria present in the milk. The sample must be collected in *sterile bottles* and must represent the *whole supply* and if it is to be examined by cultivation methods it must be *iced* and *examined promptly*. For microscopic examination *only*, it may be preserved with formaldehyde.

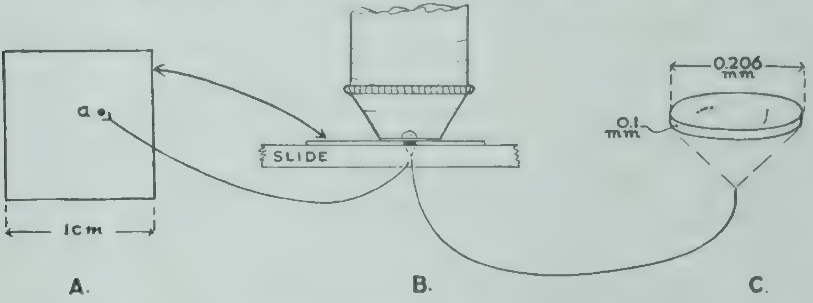

Fig. 311. The direct microscopic examination of milk.

A, Area 1 cm. square over which 0.01 ml. of milk is spread to prepare the smear. Small area (*a*) represents portion seen in one objective field.

B, Oil immersion objective focussed on milk film, showing area observed.

C, Diagram representing magnified disc of milk subtended by the microscope objective. This disc contains the bacteria in approximately 1/300,000 ml. of milk.

Breed Count. The number of bacteria in market milk is commonly estimated in health departments by one or both of two methods. In one (the Breed count), 0.01 ml. of the milk is evenly smeared on a glass slide over an area of exactly one square centimeter. This smear is treated with xylol and alcohol to remove fat and is stained with methylene blue.† A microscope, standardized by means of an object

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* The genus *Lactobacillus* is common in milk. It is more fully discussed on page 638.

† *Newman's stain for milk smears:*

Methylene blue (certified)	2.0 gm.
Ethyl alcohol (95 per cent)	60.0 ml.
Xylene, C.P.	40.0 ml.
Acetic acid, glacial	6.0 ml.

Dissolve the dye in warm alcohol. Add xylene and acid and filter. Keep in stoppered bottle.

1. Immerse smear about 1 minute.
2. Remove and drain until dry.
3. Wash thoroughly in water.

Several other staining procedures have been proposed.[7]

micrometer so that the field covers an area of approximately 1/3000 of a square centimeter (field diameter 0.206 mm.), is then used to count the visible bacteria. Using the data concerning the area covered and the volume of milk used, the total number of bacteria per ml. of milk may be computed (Fig. 311). In addition, mastitis may be detected by the observation of large numbers of pus cells and streptococci. This method is coming to be used more as a rough preliminary assay of the approximate quality of milk than as an accurate counting procedure. It is quick and inexpensive. (Compare with microscopic examination of soil, see page 600.)

Plate Count. In the second method, 1 ml. of various dilutions 1/10, 1/100, 1/1000, etc.), of the sample are placed in sterile Petri dishes. Each dish then receives 12 to 15 ml. of melted agar of a standard composition,* cooled to about 45° C., and the dish is rotated to mix the agar and diluted milk. After 48 hours' incubation at suitable temperature‡ colonies of bacteria will have developed which are visible to the naked eye or by the use of a 2× reading glass. Each of these colonies develops, theoretically, from a single organism. The number of colonies in any plate multiplied by the dilution of the milk in that plate, therefore, gives an approximation of the number of bacteria per ml. of the milk. Of course, many bacteria such as strict anaerobes, pathogens, strict autotrophs, obligate thermophils or halophils, and others with special requirements, fail to grow at the incubation temperature provided or in such media. Considerable variation in results may be expected if other media or temperatures of incubation are used.[7] The presence of certain heat-resistant streptococci, lactobacilli, etc., gives high plate counts even in pasteurized milk.

Numerical Relationship Between Breed and Plate Counts. The Breed count is usually five to ten or more times as high as the plate count because the former counts *individuals,* even those in clumps, and also *dead* bacteria, while the latter counts only live bacteria capable of developing in the medium used at the temperature used, *the clumps counting only as single bacteria* since each clump forms only *one* colony. Sometimes the clumps contain several dozen individual cells, yet they always yield only one colony. The conditions under which

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* *Agar for milk counts:*

Water	1000 ml.
Meat extract	3 gm.
Tryptone	5 gm.
Dextrose	1 gm.
Skimmed milk	10 ml.†

† For milk dilutions greater than 1/10.

Agar	15 gm.
*p*H 7.0	

‡ 32° C. or 37° C.

these tests should be done are prescribed in a manual called "Standard Methods for the Examination of Dairy Products."[3]

Grades of Milk. The actual numbers of bacteria permissible in milks of various grades vary in different cities. A good guide is the standard ordinance of the U. S. Public Health Service.[2] Various localities, as New York City, may have somewhat different standards.[9]

Abstract of milk grades from Pub. Health Bulletin No. 220 Milk Ordinance and Code of the U. S. P. H. S.

Grade A Raw Milk

Grade A raw milk is raw milk the average bacterial plate count of which does not exceed 50,000 per cubic centimeter, or the average direct microscopic count of which does not exceed 50,000 per cubic centimeter if clumps are counted or 200,000 per cubic centimeter if individual organisms are counted, or the average reduction time of which is not less than 8 hours: Provided, That if it is to be pasteurized the corresponding limits shall be 200,000 per cubic centimeter, 800,000 per cubic centimeter, and 6 hours, respectively; and which is produced upon dairy farms conforming with all of the prescribed items of sanitation.

Grade A Pasteurized Milk

Grade A pasteurized milk is grade A raw milk, with such exceptions as are indicated if the milk is to be pasteurized, which has been pasteurized, cooled, and bottled in a milk plant conforming with all of the prescribed items of sanitation and the average bacterial plate count of which at no time after pasteurization and until delivery exceeds 30,000 per cubic centimeter.

The grading of a pasteurized-milk supply shall include the inspection of receiving and collecting stations.

Grade B Raw Milk

Grade B raw milk is raw milk which violates the bacterial standard and/or the abortion testing requirement for grade A raw milk, but which conforms with all other requirements for grade A raw milk, and has an average bacterial plate count not exceeding 1,000,000 per cubic centimeter, or an average direct microscopic count not exceeding 1,000,000 per cubic centimeter if clumps are counted or 4,000,000 per cubic centimeter if individual organisms are counted, or an average reduction time of not less than 3½ hours.

Grade B Pasteurized Milk

Grade B pasteurized milk is pasteurized milk which violates the bacterial standard for grade A pasteurized milk and/or the provision of lip-cover caps and/or the requirement that grade A raw milk be used, but which conforms with all other requirements for grade A pasteurized milk, has been made from raw milk of not less than grade B quality, and has an average bacterial plate count after pasteurization and before delivery not exceeding 50,000 per cubic centimeter.

The Reductase Test. In the section on systematic studies of bacteria it was explained that actively growing bacteria bring about a lowered oxidation-reduction potential in their medium which can be detected by the use of methylene blue, for with a lowering of the O-R potential this dye serves as hydrogen acceptor and changes in color from blue to white. The test is used principally on raw milk and furnishes a rough approximation of its bacteriological quality. In performing the test 10 ml. of milk sample are pipetted into a sterile tube and 1 ml. of a standard methylene blue solution (final concentration 1:300,000) is added. The tube is closed with a rubber stopper and inverted 3 times to mix. It is placed at 37° C. in the water bath immediately. At the end of each hour during the test it is inverted once. Observations are made after 30 minutes, 1 hour, and later.[10]

The "methylene blue reduction time" is the interval between the placing of the tubes in the 37° C. water bath and the disappearance of the blue color from the milk. It is customary to consider reduction complete when four-fifths of the contents of the tube have turned white. Sometimes the reduction is irregular or mottled in distribution.

It is impossible exactly to correlate bacterial numbers and reduction time because some organisms, as anaerobes, produce a low O-R potential very quickly though present in relatively small numbers, while aerobic organisms may not reduce rapidly though present in enormous numbers. With ordinary market milk, however, the flora represents an average and reduction times are good, though rough indicators of bacterial numbers. The test is inexpensive and simple.[11]

The present status or microbiology of milk was well summarized by Robertson who said: "None of the routine laboratory procedures for estimating the number of bacteria in milk will determine whether or not infectious bacteria are present. The best assurance of freedom from infectious bacteria is that provided by proper pasteurization of the milk. The best assurance of pasteurization is that demonstrated by a satisfactory phosphatase test on the bottled pasteurized milk. The best assurance of freedom from recontamination in freshly bottled milk after pasteurization is a satisfactory coliform test in 1.0 ml. portions of the bottled product."[12]

Certified Milk. If milk is to be offered for sale unpasteurized, it is often required that it be produced only under very carefully supervised conditions. The *American Association of Medical Milk Commissions* has established rules and regulations concerning veterinary inspection of cows, sanitation of barns, utensils, etc., which may be used by health departments and milk dealers in certifying qualified farms to produce such milk.[13] It is usually called *Certified Milk* or "baby milk." The use of certified milk has much to recommend it, especially its cleanliness. It is said also to contain a larger proportion of certain vitamins essential for infants than milk which has been heated. This is a disputed point.

Most cities and states as well as the *A.A.M.M.C.* require that all persons occupied in preparing certified milk, or, indeed, any food for the public, whether it be certified milk or not, be examined periodically for typhoid, paratyphoid, and dysentery bacilli. Examinations for *Corynebacterium diphtheriae,* tuberculosis, scarlet fever and other diseases are also required for certified milk handlers. It is expensive to carry out these provisions.

Lactobacilli in Milk. The organisms of this genus are widely distributed in soil, on plants and around barnyards, and are as frequently present in market milk as *Str. lactis,* although possibly not in such large numbers. They are important in the dairy industry in several ways. Lactobacilli, as their name implies, are associated with lactic acid production. They are highly fermentative organisms, forming large amounts of acid, especially lactic acid, and have the ability to endure higher degrees of acidity than many other species of bacteria. They are therefore described as being *aciduric,* or *acidophilic*. They are not actively proteolytic. Morphologically, they are slender, pleomorphic rods (Fig. 312), often assuming coccoid or granular forms closely resembling *Streptococcus lactis* or organisms of the genus *Corynebacterium* (see page 530). They are gram-positive, nonmotile, non-sporeforming and anaerobic or microaerophilic. Several species are thermophilic and at least one species (*L. thermophilus*) grows well at temperatures as high as 62° C., a temperature quickly fatal to many non-sporeforming rods, while several other species (*L. caucasicus, L. acidophilus*) can grow at temperatures around 45° C.[14]

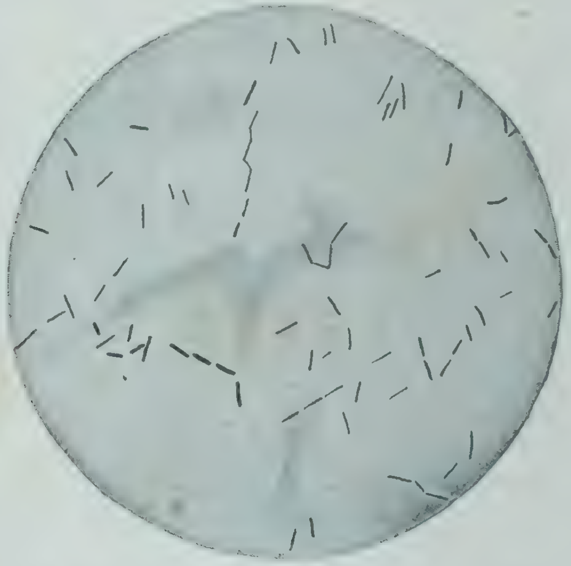

Fig. 312. *Lactobacillus bulgaricus* (× 900).

While commonly found in milk and cheese (they are important in the ripening of cheese), certain species are also found in large numbers in the intestinal contents of infants (*L. bifidus*) and in the vagina

(*L. acidophilus*), while others occur in souring vegetable products like ensilage and sauerkraut (see chapter on industrial aspects, page 691). They are rarely associated with disease but a few cases of infection due to them have been reported.[15] They grow well in milk at temperatures of from 30 to 60° C.

Fermented Milk Beverages. In certain countries lactobacilli have been used for hundreds of years in combination with certain yeasts and streptococci, to produce beverages of fermented milk. The *yoghurt* (*L. bulgaricus*) of eastern central Europe, the *busa* of Turkestan, the *kefir* (*L. caucasicus*) of the Cossacks, the *koumiss* of central Asia and the *leben* of Egypt are examples of these. In the old days, of course, the bacterial nature of these processes was unknown.

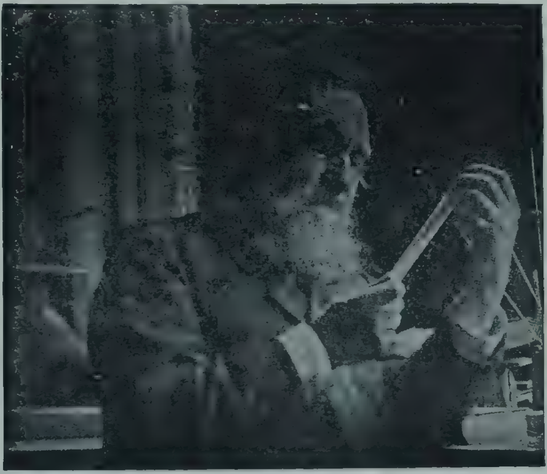

Fig. 313. Elie Metchnikoff (1845–1916). (From Garrison, "History of Medicine.")

The lactobacilli act in company with other bacteria of milk. *Kefir*, made from mare's milk, is prepared by putting "kefir grains" (small, cauliflower-like masses) into the milk. These grains consist of dried masses of lactobacilli, yeasts and streptococci (*S. lactis*) and probably other organisms held together in a matrix of coagulated casein. Their combined growth yields a characteristically flavored, soured milk. The *kefir* grains are found in the bottom of the vessels of fermented milk.

In this country, *Lactobacillus acidophilus* is used to produce a similar fermented milk product called "acidophilus milk." It is obtainable from some of the larger dairies and pharmaceutical houses.

Health Value of Fermented Milk. Metchnikoff (Fig. 313) observed that, among peoples drinking these sour milk beverages, longevity was common. He attributed this to the beneficial effects of the soured milk, and particularly to *Lactobacillus bulgaricus* or the "Bulgarian bacillus" found in *yoghurt* and *leben*. He became the foremost exponent of the idea that if these fermentative organisms could be implanted in the intestine, life might be prolonged. This is probably erroneous. However, putrefactive processes related to various disturbances like chronic constipation which results in "auto-intoxication" are probably suppressed to some extent by the high acidity produced by the fermentative action of lactobacilli in the intestine. This view is further supported by the observation that the intestinal flora of healthy, nursing infants is composed chiefly of lactobacilli (*L. bifidus*). Attempts to implant *L. bulgaricus* in the intestine, however, met with failure.

It was later found that *Lactobacillus bulgaricus* cannot grow in fluids having low surface tensions, while *L. acidophilus,* a closely related species, can. Bile produces low surface tension and it may be due to this that *L. bulgaricus* cannot be implanted in the intestine, since the intestinal contents contain bile. It was found possible to secure a considerable growth of *L. acidophilus* in the lower bowel, provided the patient consumed quantities of lactose (milk sugar) in order to provide a source of energy for the organisms. The beneficial effects of such implantations are doubted by some authorities today.

Certain lactobacilli are said to be responsible for dental decay because of the acid they produce in crevices in and around teeth. Parsons has shown that aciduric streptococci are of equal if not greater importance in this respect.[16]

Acidity of the secretion of the vagina may be due largely to the growth in the vaginal secretions of organisms formerly called Döderlein's bacillus but now thought to be identical with *L. acidophilus.*[17]

Other Bacteria in Milk. There are several rather vaguely defined groups of organisms which, like lactobacilli, are gram-positive asporogenous, nonmotile rods commonly found in market milk, cheese, butter, etc. They differ from lactobacilli in being shorter and more uniform in size and form. Some workers regard most of them as closely related to the genus *Corynebacterium, i.e.,* the diphtheroids. None is of known pathogenicity. Their nutritive requirements are complex.

The genus *Propionibacterium* includes species which are anaerobic, facultative or micro-aerophilic. They are pleomorphic and may be closely related to the lactobacilli. They are quite active in attacking carbohydrates and metabolize many organic acids and alcohols and form considerable amounts of acid, chiefly propionic, whence their name. They also form considerable amounts of carbon dioxide gas. They are of importance in producing the flavors and the "eyes" or holes in Swiss (Emmenthal) cheese. The type species is *P. freudenreichii.*

The genus *Microbacterium* includes organisms much like corynebacteria. They differ from the propionibacteria in that they produce mainly lactic acid during the fermentation of lactose, but no CO_2.

The genus *Butyribacterium* is very similar to *Microbacterium,* differing mainly in that butyric acid is produced as a principal fermentation product. It resembles *Propionibacterium* in producing CO_2. It was first found in the intestines of animals.

Transmission of Diseases by Milk. The infectious diseases most commonly transmitted by milk are typhoid fever, scarlet fever and septic sore throat, dysentery, diphtheria, tuberculosis and undulant fever.

The human intestinal infections, dysentery and typhoid fever, when transmitted by milk, practically always represent contamination of the milk by a carrier of these organisms, since cattle are not subject to these diseases. The milk must indeed be handled by very unclean people for it to become contaminated in such manner. However, it may also be infected by using typhoid-infected water supplies in the dairy. If it is not refrigerated properly, or is held at warm summer temperatures, the initial infection may increase enormously by growth of the disease organisms in it, especially if the normal milk organisms do not produce a prompt souring, which is unfavorable to most of the pathogens. The same circumstances which favor transmission of intestinal infections by milk probably favor transmission of diphtheria. That is, infection is probably introduced after the milk is drawn, by a carrier since cattle rarely or never acquire diphtheritic mastitis spontaneously. Subsequent improper storage temperatures may cause multiplication of the diphtheria bacilli if the normal flora does not overgrow them. Possibly the bovine udder may become infected with *C. diphtheriae,* but the evidence for this as an important cause of diphtheria is not very direct.

If the internal structures of the cow's udder be diseased, other pathogenic bacteria may be present in the milk in enormous numbers even as it is secreted. The organisms causing several diseases in human beings can also infect cattle and often localize in the udder. Tuberculosis, scarlet fever, septic sore throat,[17] undulant fever and possibly Q fever (see Chapter 49), sometimes result from such udder infections. Bovine tuberculosis and undulant fever are easily transmitted from animal to animal. The number and variety of organisms in milk depend upon the cleanliness of the conditions surrounding the milking process and on the *health of the cow* and of *dairy workers.*

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Chapter 38

THE MICROBIOLOGY OF FOODS

The modern human dietary is exceedingly complex and embraces a wide variety of substances from many sources and treated by various processes involving microorganisms; consequently the microbiology of foods is an exceedingly complex subject.

We shall not concern ourselves here with a rigid definition of food but will include materials generally called food and most commonly used in the dietary of normal people in this country. Since water, milk and dairy products are dealt with elsewhere, they will not be included here. This leaves us to consider other common foods, which may for convenience in discussion be grouped as: (1) Fresh foods*; meats, vegetables, fruits, fish, etc.; (2) The same foods preserved by (*a*) drying, (*b*) canning, (*c*) pickling or fermenting, (*d*) freezing, (*e*) disinfectants or bacteriostatic substances; (3) Bread; (4) Eggs.

We may divide microorganisms of foods into three general groups on a functional basis:

1) those causing spoilage or undesirable changes in the food;
2) those producing desirable changes;
3) those causing disease.

Regarding these microorganisms in each class of food it is desirable to know something of their origin or source; their numbers; their effects on the food; and how they may, if undesirable, be combatted or, if desirable, encouraged and fostered. Attempts to combat or encourage and foster microorganisms in food are the basis of industries and research in this country involving hundreds of millions of dollars annually.

* For purposes of this discussion fresh foods may be defined as those recently harvested or prepared; which are in their natural or original state, not affected by any means of preservation excepting refrigeration (not freezing) for limited periods (up to a week) and unchanged by effects of holding for sale or use beyond *slight* wilting or drying. Foods still edible but held so long that perceptibly undesirable changes in volume, weight, color, flavor, appearance or other properties of fresh products have occurred may be classed as stale. An *accurate* definition of "fresh" and "stale" is very difficult indeed, as are chemical or bacteriological determinations of these conditions. We shall use the terms in their commonly accepted meanings.

FRESH FOODS

Meat. When an animal dies of natural causes (disease, age, etc.), there is, before death, a short period (the agonal or moribund state) when there is a collapse of the defensive mechanisms which normally prevent invasion of the blood stream (and thence of the tissues) by microorganisms in the gastrointestinal tract, respiratory tract, skin and other body surfaces. Examination of muscle, liver and other tissues of such an animal immediately or shortly after death reveals the presence, often in considerable numbers, of microorganisms characteristic of the intestine, respiratory tract, etc.

If a healthy animal is suddenly killed, as in the abattoir by a blow on the head, little post-mortem invasion of the blood and tissues occurs. Cutting a large vessel with a sharp, clean knife as in killing hogs, may introduce a few microorganisms, but not many. If the animal is then immediately dismembered in a cleanly manner, as is done in well-conducted abattoirs, relatively few, if any, organisms are to be found in the depths of solid tissues. This contributes greatly to the "keeping" quality of the meat, since, if the interior is sterile or nearly so, spoilage must proceed mainly on the surface and is preventable by various treatments of the surface, such as curing or hanging in refrigerators under ultraviolet light.

Organisms which have been found occasionally *in* the tissues or organs of fresh, healthy meat include mainly micrococci, with a few gram-positive and gram-negative rods. Their origin and exact identity are not entirely clear. Their numbers are small and their occurrence not common.

The surface flora of meat depends obviously on the cleanliness with which it is handled, and the length of time it is out of refrigeration. It becomes contaminated immediately it is exposed in the abattoir. Dust from hides and hair, bacteria on gloves, hands and cutting and handling instruments, all contribute to this. The principal damage done by such organisms is to cause putrefaction, decomposition of fat, and fermentation of carbohydrates in the tissues. Some cryophilic microorganisms cause an unsightly but harmless blackening or other discoloration during refrigeration and may produce undesirable tastes and odors in the meats.

The organisms principally responsible for surface decomposition and spoilage of meat and other protein foods are bacteria of the genera *Pseudomonas*, *Bacillus* and *Micrococcus*, as well as various saprophytic Actinomycetales and molds. Many other saprophytes may also be found, as *Achromobacter*, *Proteus*, enterococci, *Escherichia*, *Aerobacter*, etc. It is to be noted that these are all organisms of soil, dust, dung, etc., and that they are aerobic or facultative.

Their numbers may be estimated by direct microscopic counts of scrapings or by cutting sections of measured area from the surface of the meat with sterile instruments, and shaking thoroughly or grinding, in a measured volume of broth or saline solution. Dilution pour plates are made of the fluid and colony counts made as for water, soil or milk.

Ground Meat. The flora of freshly ground meat is commonly much richer in numbers and types of microorganisms than that of large pieces of meat like roasts because, in the ordinary processes of preparing ground meat, scraps are used and the surface contamination is thoroughly mixed with the meat as it is ground. It is also usually warmed somewhat. To this contamination is added the flora of the grinding machine, hands, implements, dust, etc. The microorganisms present, therefore, are those initially on the surface and in addition molds, yeasts, sporeformers, Actinomycetales and others from the environment. Unless effectively refrigerated the bacteria grow throughout the whole mass and the meat spoils rapidly. If pathogens, as *Salmonella* or hemolytic streptococci are present, the meat may be very dangerous if not thoroughly cooked.

Bacteriological standards for ground meat are difficult to evaluate. According to some workers an aerobic plate count of 10 million organisms per gram of meat is a reasonable maximum. Occasional counts in ground meat of market quality may run into the billions per gram and this is exclusive of anaerobes, molds, and other organisms that do not grow under the cultural conditions provided. Decomposition soon sets in and the meat becomes putrid, rancid or otherwise offensive. The consumption of such meat is not necessarily dangerous but may cause gastro-enteritis.

The same basic principles hold true for various "prepared" fresh products such as separated fresh crab meat, shucked shellfish, flaked fish, ground horseradish, cole-slaw and the like. The more they are handled and the more thay are chopped, ground or grated, the more the surface microorganisms are mixed with the interior and the heavier the inoculation, so that such foods are much more perishable than the same foods in solid masses the interior of which is not inoculated. The acidity of products like slaw, and horseradish, mixed with vinegar, tends to retard this spoilage.[1, 2]

Fish. As is true of the flesh of mammals, the tissues of freshly killed healthy fish are sterile or nearly so. It is common practice on commercial fishing boats to "gut" fish immediately and pack them in ice. As in handling meat, the surfaces become contaminated with a variety of bacteria from the intestines of the fish, the hands and knives of the workers, the decks and cutting boards, and other environmental sources, including ice and wash water in tanks, etc. It is obvious that any contaminated products like fish, meat, milk and the like, which are rich sources of nourishment for saprophytic bacteria, will support a microbial flora, the extent of which will depend on cleanliness of environment, temperature of storage, salinity (in the case of fish), amount of handling, filleting and other factors which influence spread and growth of environmental bacteria. Failure or delay in refrigeration is the commonest cause of spoilage.

Spoilage of fish is due to much the same microbial flora as spoilage of meat, and includes saprophytic soil, marine and aquatic forms of *Achromobacter, Pseudomonas, Bacillus* and the like. Microorganisms of the sea capable of growing in albuminous material and fish slime, at salinities ranging around that of sea water (3 per cent), and at temperatures near freezing are often found on fish. These may produce spoilage in cold stored fish. Many species are phosphorescent and fish spoiled by these organisms may glow in the dark.[3, 4] Anaerobes (*Clostridium*) which cause foul smelling decomposition apparently originate from feces of the fish and from sea bottom mud. Chlorinated water is often used to wash fish as they are gutted and scaled, with the object of preventing microbial growth. In storage some use has been made of ice containing calcium chloride with reportedly good effect.

Oysters. These and similar shellfish under market conditions are often rather heavily contaminated with dirt from the shells and from the benches at which "shucking" (removal of shells) is done. The flora includes marine forms similar to those on fish, and organisms from soil and dirt from handling. If the water in which they are grown has a rich bacterial flora, as is rather apt to be the case,* the shell liquor may contain considerable numbers of various sewage bacteria, including the coliform group.

In good commercial practice oysters are held (floated) for some days in clean water, either natural bay or sea water or tanks of pure chlorinated water. Floating in fresh water removes the desirable salt and flavor and the oysters swell up because of osmotic pressure. Floating in clean water permits the shellfish to rid themselves naturally of much of the contamination inside the shell and helps remove dirt on the outside.[5] Oysters in the shell should be well hosed down with clean water before shucking and in good places cleanliness of workers and surroundings are insisted upon.

Tests for the coliform group of bacteria in shell liquor, or the liquid around shucked oysters, are made much as tests are made for this group in drinking water. The results are expressed in a similar manner.[6]

Perry and Hajna recommend the use of a modified Eijkman test to detect fecal *Escherichia coli* and eliminate unnecessary condemnation of oysters containing innocent contamination with the environmental, mainly soil-living coliforms (*Aerobacter* and *Esch. freundii*). These do not as a rule form gas in the Perry and Hajna medium.[7] This principle has much to recommend it, but not all workers are in agreement as to the accuracy of the method.

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* Oysters fatten best on sewage, and have long been known as vectors of typhoid fever if taken from polluted waters. Typhoid bacilli may live for two weeks or longer in live and shucked oysters. State and Government supervision of the beds has virtually removed infected oysters from the market. Formerly common, typhoid due to oysters is now very rare.

Fruits and Vegetables. The principles underlying the microbiology of fresh meat and fish products apply equally with regard to fresh vegetables and fruits. That is to say, the internal tissues of whole healthy plants are free from bacteria but contamination of the surfaces by microorganisms from the soil, hands, packing machinery, packages, etc., can and does occur. The nature of this contamination obviously will be governed by its source and the mode of handling the product. Vegetables growing in the soil, such as root crops, have adhering to them saprophytes of the genera *Bacillus, Pseudomonas, Micrococcus, Clostridium, Lactobacillus, Enterococci,* Actinomycetales, as well as many species of yeasts, molds, and soil protozoa and many others, depending on the nature of the soil, whether heavily manured, boggy or sandy, and the like. These organisms are usually held in check by *drying,* exposure to sunlight of the surface of the vegetables, and by refrigeration. Intact vegetable skins resist invasion of microorganisms.

Vegetables of any sort soiled with *moist* earth, kept in a warm, humid, dark place will decay rather promptly, depending on the kind of vegetables; whether they are bruised and scratched or have intact surfaces; are juicy or not. Hard, nonsucculent vegetables, such as turnips and potatoes, with whole skins, will stand storage better than damaged ones oozing juice. Soft, succulent ones like lettuce, asparagus and spinach which offer good pabulum to microorganisms, soon decay. Vegetables like lettuce and cabbage, beans and broccoli, which grow above the soil, likewise have soil bacteria upon their outer surfaces but in lesser numbers. Corn on the cob, beans, and vegetables protected from contamination by the husks, outer leaves, or other natural coverings are fairly free from gross contamination. The interior of a tight head of lettuce or cabbage, while not sterile, will not decay as soon as the outer leaves. In general, undamaged vegetable coverings and skins offer a great barrier to the entrance of bacteria of decay, much as intact skin protects an animal against infection. Dry vegetables stored in a cool, well ventilated place will keep much better than damp vegetables or those stored in damp, warm, close rooms.

Fruits grown on trees, like apples, pears, cherries and peaches, will keep fairly well if not damaged, but microorganisms gain entrance through broken stem attachments, bruises, and insect bites. Unless the fruits are picked before they are ripe (when they are more acid, have less free juice, less fermentable sugar and are harder than ripe fruit) they soon decay if not refrigerated or preserved. Here again soil and environmental microorganisms play a major role, being brought to the fruits by dust, insects, handling, etc.

Soil fertilized with fresh human excreta, as is customary in the Orient, Egypt, and elsewhere, contaminates fruits and vegetables with intestinal organisms among which may be *Salmonella typhi* and amebae of dysentery (*Endamoeba histolytica* cysts). These organisms may be found on the products, and outbreaks of enteric disease have been shown to be due to this source of infection. Hands soiled with infectious feces may also contaminate fruits and vegetables, as well as other foods, in the kitchen. *S. typhi* may survive in moist garden soil for periods long enough for crops like lettuce, spinach and radishes to mature and be served on the table.

Eggs. Steps in the formation of an egg within the hen have been outlined in Chapter 7. If yolk is formed in an infected ovary, or albumen is laid on in an infected oviduct, or if other parts of the egg are formed in infected organs, then it is not difficult to account for the presence of bacteria in newly laid eggs. Certain pathogens, especially *Salmonella pullorum,* which is common in many henneries, can infect the avian tissues and has been shown to occur in eggs and in food products made with those eggs and to cause outbreaks of disease (see page 723).[8] Other organisms, not human pathogens but capable of causing "rot," are also said to be incorporated inside eggs before they are laid. While there appears to be some difference of opinion on this point most data tend to support this view.

The question of organisms inside of intact eggs appears to be one not easily answered. There may be several sources of error. The most subtle source of error in determining the presence of microorganisms in eggs is, of course, their introduction from the shell at the time of opening the egg for examination. This depends entirely on the intelligence, skill and carefulness of the operator. The pouring of eggs through a hole in the shell invites contamination. The contents should be drawn up in a pipette or syringe after making a minimal-sized opening in the portion of the shell sterilized by burning it with a needle-blast flame. Application of surface disinfectants may fail to reach microorganisms in the pores of the shell. It is also very difficult to obtain contents of the yolk sac without introducing contamination from the white which always surrounds it.

Experience of those using eggs for the cultivation of viruses and other organisms seems to indicate that fresh, clean, sound, fertile eggs do not as a rule contain bacteria that grow under the incubator conditions generally used. If eggs generally were contaminated intrinsically, there would be much more trouble from this source. Even infertile eggs are mainly found in good condition after one to two weeks' incubation.

It would be of interest to know the effect of development of the embryo on microorganisms originally inside the eggs. In the case of some bacteria, as avirulent diphtheria bacilli, the chick hatching from an experimentally infected egg may continue to harbor the organisms. This appears to be true also of *Salmonella pullorum* under natural conditions of infection. The growth of some viruses and pathogens in fertile eggs kills the embryo.

As soon as an egg is laid the surface becomes heavily contaminated. If kept *cool* and *dry* the surface microorganisms cannot grow extensively. They are prevented from entering the egg for some days largely by the natural, dry, mucilaginous coat on the surface. Various organisms can rapidly penetrate the shell if the mucilaginous coating of the shell is removed by washing, long storage, action of enzymes, or by mechanical means, such as cleaning the egg with sandpaper. These eggs do not keep well in storage, either cold or "water-glass."

Molds can grow on the outer mucinous coating if eggs are stored in humid atmospheres (90 to 100 per cent saturation) at ordinary climatic temperatures. These may give the eggs "off" odors and tastes as well as unsightly appearance and may penetrate the shell and contaminate the interior. They are frequently found in spoiled eggs. Eventually, at temperatures and humidity suitable for their growth, this protective film is decomposed and the interior of the eggs is invaded with consequent decay, the rate depending on temperature and numbers and kinds of microorganisms.

The cleanliness of nests and birds, of hands, of containers and of storage places all affect the keeping quality of the eggs because they influence contamination of the shell. The principal sources of contamination are fecal matter brought to the nests on feet and feathers of the birds, and mud in wet weather, if they run in yards.

The flora of eggs, then, is largely of fecal and soil origin and includes the coliform group, *Pseudomonas, Proteus, Clostridium, Bacillus, Micrococcus, Achromobacter* and the like as well as molds, and yeasts and actinomycetes. This is very similar to the flora of milk. The type of rot in eggs depends largely on the predominating organisms; usually a mixed flora is found. Most of the rot organisms are proteolytic. These can therefore attack the mucilaginous coating.

Egg Products. When eggs are broken under commercial conditions the contents are contaminated with the shell flora and microorganisms of the openers and containers. The bacteriology of commercial egg products is therefore largely that of the shells and of the environment. In commerce, the best (freshest) eggs are sold in the shell while mainly stale or even spoiled eggs are used for liquid, frozen or dried products. The flora of the latter products is therefore sometimes rather rich. Plate or direct microscopic counts of such egg products may run from one million to one billion or more per gram. This does not necessarily make the eggs unfit for use in cooking any more than high bacterial counts destroy the commercial value of lower grades of milk. If the egg products are not promptly frozen, refrigerated or dried, however, the heavy initial contamination quickly multiplies and ruins the product. The addition of sugar or salt may retard this process to some extent. Storage of egg products at low temperatures appears to reduce initially high bacterial counts, especially if salt is added.[9]

Egg white is often sold separately as a dried product. This contains a considerable amount of fermentable carbohydrate which makes the whites more perishable. It is now customary to permit a preliminary fermentation of the whites by means of organisms of the coliform group or, much better, by yeasts which do not attack the protein. A small amount of some nutrient material for the yeast, such as yeast extract, may be added to initiate a good growth of the fermenting organisms.[10] The fermentation proceeds for about forty-eight hours at about 28° C. This removes the carbohydrate under controlled conditions and greatly increases the keeping quality of the whites. The product becomes more acid if the bacterial fermentation occurs. If large numbers of proteolytic organisms, as *Pseudomonas* or *Serratia*, contaminate the batch, it may be ruined.

Bread. Since bread is a mixture of several ingredients its microbiology is largely that of its constituent parts: flour, milk, water, salt, sugar, shortening, leaven or yeast cake, etc., and mixing apparatus. Flour and milk normally contribute most of the flora, though if dirty salt, water or shortening are used these may contribute a large share.

Bread made of "sour" dough (Sauerteig), and self-rising or salt-rising breads are leavened by gases formed by bacteria, mainly of the coliform group and mixed fermentative organisms, probably including yeasts. These are introduced usually from previous lots of sour dough and are a fortuitous mixture from environmental sources, as a rule. The most agreeably flavored sour-dough breads contain lactic acid and other products of bacterial fermentation, as well as diacetyl, a substance giving pleasant aroma to butter. These are produced by lactobacilli and by lactic streptococci, etc.

Many of the organisms in ordinary baker's bread appear to come originally from the flours or meals used in them. Bacterial counts of flour range from a few hundred thousands to several millions per gram. These are mainly organisms of soil and fields. Humidity and temperature of storage as well as original cleanliness of the grain, of the mills and storage places affect the microbiological content. Many molds occur in flour and can cause spoilage in the stored product, especially under humid conditions too dry for bacterial growth. During baking most of the molds, yeasts and non-sporeforming bacteria are killed but some spores and conidia may survive.

Ropy Bread. If bread is made with flour or other ingredients containing large numbers of spores of *Bacillus mesentericus* and related species these may well survive baking and grow in the bread, producing a mucinous slime. When the bread is broken apart this slime is drawn into long threads resulting in a defective product called "ropy bread."

Moldy bread is usually the result of extraneous contamination of cut surfaces, or crust, by hands, dust and knives, although mold conidia in the ingredients may survive slack baking and grow in the interior of the bread. Moldy flour is an important source of the conidia. Thorough baking kills most mold conidia as they are not much more resistant than vegetative cells. Slight surface growth of mold does not necessarily destroy the bread but gives it a musty odor or taste.

Since bread is not sterile it should be cooled promptly after baking. The wise housewife keeps bread in the refrigerator in warm weather. Wrapping bread in waxed paper helds keep the bread clean and prevents drying out but conserves a humid atmosphere and favors growth of molds and bacteria. Pathogenic bacteria cannot survive proper baking of bread but may be introduced after baking by unsanitary handling.[11, 12]

PRESERVED FOODS

The preservation of foods has for its main objective the total elimination, or prevention of growth, of microorganisms capable of causing decomposition; those producing undesirable odors, tastes or discolorations; and the elimination of those capable of causing disease.

Methods may be divided into two general classes: (1) Bactericidal methods, resulting in the immediate sterilization (or "virtual sterilization") of food; (2) Bacteriostatic methods, which restrain the growth of microorganisms for long periods but do not, under the usual conditions, sterilize the food. From a practical standpoint, canning or such processes as making jams, jellies and preserves, chemical preservation, and smoking, are the principal methods of the first type; drying, freezing, refrigeration at temperatures slightly above freezing and various types of pickling, including fermentation, are the most important bacteriostatic procedures. As has previously been pointed out, bacteriostasis, if prolonged, results in death of the organisms.

Canned Foods. Modern home or commercial canners have to consider not only the killing of bacteria likely to cause spoilage or disease but also the effect of the processing on the palatability and appearance of the food. If canned foods are processed long enough they can be absolutely sterilized. This may require so many hours of heating in steam under pressure that the palatability of the food often suffers and it may become mushy and discolored and have a bitter flavor. Prolonged heating also adds to the cost and trouble of canning.

The aim of the canner, then, is to heat as little as is possible, consistent with safety from botulism (food poisoning) and spoilage.[1, 12, 13, 14, 15, 16]

From the standpoint of disease, the only organism likely to be present in food and to resist processing (autoclaving) temperatures is *Cl. botulinum* (see page 527). Even the most resistant spores of this species are killed at 115° C. in ten to fifteen minutes. Thus, if the *whole* of a mass of food, *including the center of cans,* neutral in *p*H reaches 115° C. and is held at that temperature for fifteen minutes, danger of botulism and of food infection due to any non-sporeforming organisms is apparently nil.

A good margin of safety in time or temperature or both must be left for the possibility of some super-resistant or protected spores. Actually, in order for the center of solid masses, like meat and sweet potatoes in a can, to reach 115° C., the whole can must be held at that temperature or higher for a considerable time—possibly an hour or two hours, depending on the size of the masses and of the can and the amount of circulation of fluid in the can, as well as insulating air pockets. In commerce, pockets are largely eliminated by evacuation. Also, the food is already hot when placed in the cans. Heating processes affect the *nutritive* value of some canned foods, especially vitamin content. This is well summarized by Wagner et al.[17, *] Actual times and pressures to be used in home canning can be obtained from various Farmer's Bulletins on Home Canning, available from the U. S. Superintendent of Documents, Washington, D. C.

Botulism. In meat sausage, especially the softer, moist sausages, as well as inside of cans of meat, vegetables and fish, strict and facultative anaerobes may find good pabulum and good anaerobiosis. The bacteria get into the containers when dirty foods, having soil or manure on them, visible or not, or unclean containers, are used. Sometimes the growth is not sufficient to spoil the food and it may be eaten.

Of all the bacteria that get into canned goods and sausages, *Clostridium botulinum* is by far the most dangerous. Like *Cl. perfringens* and *Cl. tetani,* it gives off a very potent exotoxin, which is excreted into whatever material encompasses it as it is growing. Unlike the toxins of *Cl. perfringens* and *Cl. tetani*, however, the toxin of *Cl. botulinum* remains poisonous when swallowed, being apparently un-

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[*] New methods of heating large masses throughout in a few seconds by radiation with penetrating infra red heat waves will probably revolutionize the food processing in a few years. The passage of high voltage electrons is said to sterilize food.

affected by the acidity or pepsin of the gastric juice as are most other bacteria and their toxins. Botulinus toxin is absorbed directly from the stomach and intestines. It affects the nerve-muscle complex, producing a flaccid paralysis, particularly of the face, eyes and throat. As in tetanus, after symptoms appear antitoxin is of little avail therapeutically and, as no one knowingly eats food containing botulinus toxin, prophylactic antibotulism serum is never used.

Botulinus toxin may be of three serological types, A, B or C. Type A is the most common in the United States; type B not common. Type C does not occur in man. There is a specific antitoxin for each type. The toxins are highly pathogenic for mice and other animals as well as man.

Botulism has a high death rate, 68 per cent. Many cases of botulism and also *Salmonella* and *Staphylococcus* poisoning (see page 538) in the past were called by the common, incorrect name of "ptomaine poisoning" (see ptomaines, page 663).

One may easily guard against botulism by boiling all canned foods for twenty minutes after opening them. The toxin of *Cl. botulinum* is destroyed by this amount of heating. Acidity greatly reduces the time and temperatures necessary to preserve foods by heat. Canned tomatoes, rhubarb, and acid fruits, for example, require only a few minutes at 100° C. to preserve them. Neutral materials like corn, spinach, and beans require much longer periods at higher temperatures. *Cl. botulinum* will not grow if foods have an acidity greater than *p*H 4.5. Some foods, ruined by long processing, are therefore acidified to a greater degree than *p*H 4.5 and processed for a much shorter period. The acidity not only reduces danger from botulism in the stored food but makes the short heating more effective.

Acid foods like tomatoes, pickles, most acid fruits, etc., seldom cause botulism. Jams, conserves, jellies and sugar syrups likewise do not support good growth of *Cl. botulinum* because of the high osmotic pressure of sugar solutions. The four home-canned foods most commonly responsible for botulism are corn, beans, beets, and asparagus. Note that three of them are often contaminated with soil.

Precautions in Home Canning. In home canning, much can be done toward obtaining safer, more palatable and better looking foods if a few simple precautions are taken:

1. Use only sound foods, fresh as possible, well washed and clean. They should be boiled in a covered vessel, with just enough fluid (water or juice) to cover them. This should continue for at least fifteen minutes, or until they are heated throughout. This will kill many spores.

2. With a clean ladle, transfer the hot food into clean (boiled) preserving vessels and cover as directed by the manufacturer of the container. Do not pack too tightly. Fluid must circulate freely.

3. Place the vessels promptly, while hot, in the sterilizer. This should preferably be a pressure cooker. If fractional sterilization (tyndallization) is to be used, any covered vessel, such as a wash boiler, may be used. The water in the sterilizer should be boiling.

4. Manufacturers' directions generally accompany pressure cookers. If you have no directions, then, for general purposes, proceed as follows:

(*a*) *Be sure the safety valve, air vent, and pressure gauge are in working order or you may have a bad explosion.*

(*b*) Open the air vent wide. Have about one inch of water in the bottom of the cooker.

(*c*) Clamp on the cover tightly in the correct position (see arrow on cooker and lid).

(*d*) Leave the air vent open until *pure steam* issues with a *loud hissing noise*. Allow four minutes after this.

(*e*) Close the air vent and watch the pressure gauge till from 12 to 15 pounds pressure is reached.

(*f*) Adjust the heat source so that the pressure neither rises nor falls during at least thirty minutes (for quart jars and smaller sizes).

(*g*) At the end of the time of sterilizing, turn off the heat and *allow the pressure to subside completely*. Do not open the air vent until the pressure is down.

(*h*) Open the air vent.

(*i*) Remove the lid and the food vessels and tighten any loose covers.

Acid foods like tomatoes need not be processed as long as neutral foods like corn, peas and spinach, since the combination of acid and heat is more rapidly bactericidal than heat and a neutral reaction. Foods in large masses, like meat, should be processed longer in order to allow the heat to penetrate the interior. All foods should be covered with fluid. Don't fill jars over three-quarters full, or they are apt to boil over.[18, 19]

Contamination with soil is especially to be avoided as it introduces thermophilic bacteria likely to cause spoilage in spite of heating, as well as *Cl. botulinum*. Some thermophilic sporeformers are among the most heat resistant microorganisms known. One such, known as *Cl. thermosaccharolyticum* (No. 3814) is an anaerobe and may cause spoilage of moderately acid, processed foods if they are not promptly cooled after heating or are stored at warm temperatures at which the thermophil can grow. *Cl. sporogenes* (No. 3679), a putrefactive anaerobe much like *Cl. botulinum*, but harmless, will cause spoilage in neutral or slightly acid foods stored at around 30° C. It is killed by the usual processing. Spores of some other organisms are much more resistant. For example, *Cl. stearothermophilum* (No. 1518) will resist any ordinary processing, being able to withstand 115° C. for over two hours at *p*H 7.0. However, being a thermophil, it does not grow if the processed cans are promptly cooled and stored in a cool place.

Canned foods may frequently contain spores of such organisms, which are viable but fail to grow under ordinary conditions of storage. These foods may be said to be "virtually sterile" or "preserved by heating" or "commercially sterile." Prolonged storage in warm places may cause such foods to spoil, but under usual conditions they remain sweet and good for sufficient periods. *Cl. stearothermophilum* and a number of similar species, when they grow, produce a souring but no gas, so that the ends of cans are not bulged by pressure, but are flat. Hence these bacteria are called "flat sour" organisms among canners. They occur in foods like corn, spinach, etc. (non-acid). These organisms are much used in research in canning. The subject of thermophils is well reviewed by Gaughran.[20] Spoilage organisms which produce gas in cans cause the ends of the cans to bulge. In the industry such cans are called "springers" or "hard swells," depending on the amount of gas pressure in the can.

Freezing. The frozen food industry is now a very important one.[21, 22, 23] While some microorganisms in foods are killed by freezing, considerable numbers survive and a few grow at or below freezing temperatures, but do so very slowly. Intestinal pathogens, if present, will survive freezing for weeks. Most foods remain practically unchanged for weeks or months while frozen at about —26° C.* On thawing they are subject to rapid deterioration because the tissues are dead and disrupted and the natural resistance of live tissues is lost. Every up-to-date food store now has its frozen-food cabinet and many homes and communities have frozen food lockers. As in canning, only whole, sound, clean, fresh foods should be selected. Freezing *must be prompt and rapid* to be effective. The frozen foods are virtually the same as fresh foods so long as they remain frozen. The freezing must be *rapid* to eliminate separation of tissue fluids, changes in color, flavor and consistency, etc. Much of this change is due to drying, oxidation and formation of unduly large ice crystals, but may be due in part to growth of microorganisms if the freezing is too slow. It is of especial importance to note that, if frozen foods are once thawed, they may undergo *rapid* microbial decomposition. Once thawed, they should be used immediately. Interruptions to electric current supplying frozen-food lockers may cause serious damage if prolonged for twenty-four to forty-eight hours or more, since microorganisms, including dangerous pathogens like *Cl. botulinum,* may grow while the food is thawed and their activities may not be apparent when the food is used after re-freezing. Indeed, food frozen after being thawed unknown to the consumer, may be a very dangerous product.[24, 25] The freezing of cooked foods goes far to eliminate dangerous organisms and rapid spoilage after thawing. A means of making any food safe from infectious organisms and from botulinus toxin is to boil it for at least fifteen minutes.

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* This is the temperature of the home freezing locker.

Refrigeration. Most microorganisms can grow only very slowly at around 10° C., the temperature of the average household electric refrigerator, but few are killed rapidly by such temperatures. The method is an excellent example of bacteriostasis. The use of the home refrigerator has given almost everyone some personal experience with the usefulness of near-freezing temperatures. Foods of the most perishable sort will remain edible and unspoiled for days or even weeks in the refrigerator, but eventually develop sour, offensive tastes and odors and undergo slow decomposition. At about 10° C. and below 15° C. most foods will remain safe from botulism and in good condition for many days, even up to a month. Every refrigerator should have a thermometer in it, as an inefficient machine not only wastes food and ice or electricity but may give a false sense of security from food poisoning. The thermometer is for consultation and guidance, not for decoration.

Foreign Substances. Many so-called chemical preservatives were formerly used to check microbial growth in foods. Formaldehyde, salicylates, boric acid, hypochlorites and most others have been prohibited by law as undesirable or harmful. The two most commonly in use today are sodium benzoate and sulfur dioxide or sulfite. Probably neither is harmful in the concentrations used (0.1 per cent benzoate and up to 3,000 p.p.m. sodium sulfite). They act mainly to prevent growth and do not necessarily sterilize food. Benzoate is said to be especially effective in acid foods. The sulfite also preserves color of dried fruits. Benzoate is used in catsup and some other vegetable products which are not heat processed, as cider, etc.

Sodium nitrate or nitrite, or mixtures of these, are another form of preservative usually added to salt (NaCl) curing mixtures for meats (corned beef, ham, etc.). Whether the nitrates or nitrites have any bactericidal or bacteriostatic action per se seems to be undecided, but practical meat packers find much less spoilage when these salts are used. They appear to have an adjuvant action in acid solutions (most cured meats are acid, *p*H around 5.8) and on the preservative effect of NaCl. Nitrites also help to give good red color to meat.[2]

Smoking. This is a time-honored means of preserving meats and fish. It is not much used for vegetables or fruits. The preservative principles in wood smoke are various cresols, a mixture of formic, acetic and other organic acids and alcohol ("pyroligneous acid"), and formaldehyde; all of which are bactericidal substances. These are gradually absorbed in small quantities by the tissues exposed to the smoke and the meat is "cured" and rendered impervious to the action of most microorganisms, as well as given an agreeable aromatic flavor. Kept dry, it is preserved almost indefinitely. In warm humid weather it may become moldy and rancid, especially on the surface, due to mold growths.

Drying. This method of preserving food is perhaps as old as the human race. It is well exemplified by making hay, raisins, etc. Exposure to the sun adds the effect of ultraviolet to such procedures. Drying per se is a bacteriostatic method and dried foods, unless preserved with such materials as salt, sulfite, etc., are not stable in humid conditions. The flora is much the same as that of fresh foods. The production of dried egg powders, dried milk, dried fish, fruits, etc., are large industries. The problems in this field are summarized by the Committee on Food of the American Public Health Association,[26] and by Goresline.[27]

Preservatives Commonly Classed as Foods. *Salt.* The smoking process mentioned above would often be too slow in its action to prevent spoilage of fresh meat or fish, especially in warm weather, unless the tissues are shredded or cut in strips to facilitate contact of the smoke, or unless smoking is done at low temperatures or after salting, etc. For large pieces the preservative action of saline solutions of high osmotic pressure are used to prevent early spoilage. For hams, fish, etc., to be smoked, salt is applied either in the form of strong (20–25 per cent) brine in which the foods are soaked for some days, or in the form of a dry mixture (also containing sugar and flavor) which is rubbed into the ham or fish—salted fish, "sugar-cured" hams. The sugar and salt diffuse through the meat, withdraw much fluid and cause the meats to shrink and become drier and firmer. The color is also fixed. Growth of microorganisms is inhibited by the salt and sugar concentration. Often the salt-sugar or brine solution is injected with trochars into the tissues, especially along the bone of hams. Here "bone-taint" is prone to occur due to bacterial action at the center of the piece of meat farthest from the surface action of chilling, curing or smoking. Pickling solutions may also be injected through the vascular system of the animal after slaughter, before it is cut up.

If large numbers of halophilic or other bacteria are introduced by contaminated pickle solution or instruments, the entire mass may spoil, sour, become gassy, rancid or putrid, depending on the kinds and numbers of organisms introduced. Dirty salt will of course introduce heavy contamination to any food receiving it. Cleanliness in food packing plants pays big dividends in money saved.

Halophilic organisms often grow in salt brines used for preserving foods. This includes yeasts, mold and bacteria. Some of these cause desirable flavors or they may cause putrefaction.

Some workers believe these halophilic organisms to be merely variants derived from ordinary saprophytic species of soil water, dust, etc. by a process of adaptation to higher osmotic pressures. There is considerable to support this view. In many industrial processes, as in food processing, there appear to be microorganisms peculiarly adapted to grow under the conditions of that process, and many have names and identities which are familiar mainly to workers in that industry. Examples are the organisms inhabiting meat pickle vats, the thermophils in fermentation vats in fermentation industries, the cryophilic organisms of refrigerator plants, and so on.

The action of the salt and sugar would be too late to prevent early spoilage of large pieces of the meats, etc., were they not first thoroughly chilled. For this reason sugar and salt curing are usually done in the winter or in refrigerators.

Sugar. The use of sugar syrups in preserving fruits has been discussed in the section on bacteriostasis and needs little further comment here. Its use in curing meats (sugar-cured hams) has been mentioned in this chapter. To be effective, the osmotic pressure of solutions of sugar or salt must be greater than that of the microorganisms. Sugar solutions of 50 to 70 per cent concentration effectively prevent most growth. A very few organisms (some yeasts and molds) may grow slightly in such material. In humid atmospheres the higher concentrations of sugar may absorb enough water from the air to permit surface growth of molds.

Many microorganisms may survive for long periods in such solutions and in honey, molasses, etc., and may even grow slowly to some extent. In preserving foods with syrup it is better to heat the syrups and scald the fruits, or heat both together thoroughly, to kill the bulk of the microorganisms initially present. Exclusion of air, as by paraffine or tight covers on jars of preserves will prevent growth of adventitious aerobic organisms, especially molds, on the surfaces.

Spices. Spices used for flavor are often heavily contaminated with soil and dust flora. Most spices have no inherent bactericidal or bacteriostatic activity, in spite of a centuries-old tradition to that effect.

Acids. Acids naturally occurring in foods as a result of natural fermentation or souring are, principally, lactic as in cheese, buttermilk, etc.; and acetic as in vinegar. Usually there are present also in these products small amounts of various alcohols, glycerine and some other acids. Acids in ripe tomatoes and fruits include citric, malic, tartaric and the like. All have a preserving action except against certain aciduric molds and organisms such as lactobacilli. It is not alone on *p*H that these acids depend for their protective action but upon the anion and especially the whole, undissociated molecule as well (see

Chapters 6 and 11). Acetic and tartaric acids at a given *p*H, for example, are less toxic for a number of microorganisms like *E. coli* than is citric acid at the same *p*H. The situation may be reversed in the case of other species.

Lactic and acetic souring of foods is due largely to lactic streptococci and lactobacilli, but undesirable flavors may be produced by concomitant growth of soil contaminants, coliform organisms and other fermentative species. Foods like sauerkraut which rapidly sour with the formation of an edible preservative acid, such as lactic or acetic (*p*H about 4.0), are not likely to spoil rapidly due to overgrowth of other organisms such as those causing putrefaction or other spoilage. Few of the latter microorganisms can grow rapidly at the *p*H of fermented foods like buttermilk, cottage cheese, sauerkraut and vinegar. Any slight rise in temperature makes this acidity very lethal for bacteria.

However, there are some streptococci and other aciduric and acidophilic organisms, genus *Bacillus,* molds, etc., that can grow in naturally fermented products, attacking the acids and proteins and producing alkaline growth products, carbonates, amines, etc., which soon alter the *p*H so as to permit putrefaction to set in if the foods are not refrigerated.

Foods in the Kitchen. Aside from considerations of cleanliness, sanitation and the proper processing of preserved foods, which have as one of their main objectives the prevention of disease, the domestic economist is interested in saving from spoilage foods which are kept from one meal to another—the pot of good soup (excellent bacteriological medium!) which is saved for tomorrow's dinner; the leftover porridge or bread pudding, and so on. All are subject to spoilage by microorganisms from dust and utensils unless those organisms are killed or kept from mutiplying.

The household refrigerators or freezing cabinets offer the readiest means for saving foods. When these places are overcrowded, or are for other reasons not available, spoilage can be retarded by reheating foods in covered vessels and not opening the vessels until more of the food is needed (Tyndallization). For example, the pot of soup, stew, vegetables or other non-acid foods may be brought to a boil for a few minutes in a vessel with a good dust-tight cover on it. The heat kills any vegetative forms of microorganisms and the spores of "wild" yeasts and molds that might have gotten in while it was cool and uncovered. A good many bacterial spores will be killed also, but some will survive. However, these probably will not grow sufficiently to cause spoilage for twenty-four hours or more. If the soup should be brought to a boil the next day, the cover not having been removed in the meanwhile, then virtual fractional sterilization has been accomplished and the food should keep for some time. The importance of recooking as a means of preventing certain kinds of food poisoning and food infection has been summarized by Hussemann and Tanner,[28] Getting, et al.[29]

On solid foods not immersed in water, such as a roast of meat, spoilage tends to occur mainly on the surfaces. Clean utensils introduce less contamination than soiled ones. If the roast is heated in a very hot oven for a few minutes after the meal, so as to cook only the exterior, and is left in the oven without opening the door, thus avoiding re-contamination from the air, this surface growth is definitely checked and the meat may remain good for some days, depending on the extent and depth to which initial contamination was introduced, and the temperature of the kitchen. In reheating meat, bowls of pudding, etc., in this manner it is desirable to use a "self-basting" roaster or other covered dish to prevent drying out of the food. The reheating should be for a short period, say ten to fifteen minutes, at high temperature.

Bacteriological Examination of Foods. The manufacturer of foods like cheeses, sauerkraut, and butter in which the action of certain species of microorganisms is essential to flavor, consistency and other special properties of the product, is interested mainly in those certain species. The sanitarian is interested in pathogenic species such as certain groups of hemolytic streptococci in milk, or enteric organisms (dysentery and paratyphoid group) in food suspected of causing an epidemic of gastroenteritis. His methods are largely those of the diagnostic bacteriologist and are primarily qualitative.

The domestic, as well as the sanitary, microbiologist is interested in cleanliness, spoilage, and keeping quality and these usually involve mixtures of mainly non-pathogenic forms derived from the environment (dust, utensils, etc.). The most important considerations are their numbers and the rate of their development in the food. These are directly related to cleanliness, keeping quality and spoilage. The methods of the domestic economic microbiologist are, therefore, largely quantitative. However, he is also often especially interested in the presence of the coliform group, because of its implications with regard to fecal or dung contamination, soiled hands of food handlers, etc. In such case he uses methods similar to those used for the detection of this group in milk or water. This is qualitative as well as quantitative bacteriology.

Quantitative Microbiology of Food, such as estimating the total numbers of bacteria in milk, water, ground meat and the like, involves basically the same procedures regardless of the product. The main differences in procedures lie in collection, preparation and measuring of the sample. These steps are guided by the nature of the material. So many different kinds of food are examined microbiologically that detailed methods for all of them cannot be given here and the student is referred to literature on specific foods (see list of references) for exact procedures.[1, 2, 30, 31, 32] In general it may be said that the plate-count method for examining milk (see Chapter 37) is the prototype of quantitative methods for examining fluid food products in general; while the coliform examination of water (see Chapter 34) is the type of qualitative procedure applied in examining foods for this group.

The nature of the medium used, the temperature (thermophilic or mesophilic) and conditions of incubation (aerobic or anaerobic) and other factors (acidity, salinity, etc.), must be modified to suit the flora being counted. No single process gives a complete count of a mixed flora. The errors are mainly those inherent in the counting of organisms in milk and water. The plate-dilution count may be made both quantitative and qualitative by using a medium, incubation temperature or other factor selective for only one species or group of species. For example, acid-formers may be detected by adding glucose and an indicator to the nutrient medium used for plating. The numbers of bacterial spores present may be estimated by heating the material to 80° C. for twenty minutes before plating. Other selective procedures will occur to the ingenious bacteriologist and can be adapted to various problems.

In examining solid foods such as fruits or meat, scrapings of the surface—often of exactly measured areas and to a specified depth—are collected, emulsified or shaken in sterile water or saline solution and a plate count made of the fluid. In most instances an attempt is made to transfer the microorganisms from the material being examined as completely as possible to a liquid which is then handled by dilution and plating or otherwise. The quantitative data thus obtained are rough estimates but are often very useful.

Other methods for solid foods involve completely homogenizing the food by very fine grinding or chopping as in a Waring blendor. This liberates microorganisms in the interior parts so that they may be suspended in fluid, diluted and plated. Instead of plating, tubes of fluid medium, possibly with fermentable substances and gas tubes, may be used for the dilutions, to count organisms capable of forming acid or gas or both, or merely turbidity, in the medium furnished.

In dealing with foods like ground meat, crab meat, diced vegetables, etc., weighed or measured samples are thoroughly shaken or homogenized with water and saline and the fluid plated or cultured in serial dilutions. Counts are expressed in microorganisms per unit of weight or volume of the food.

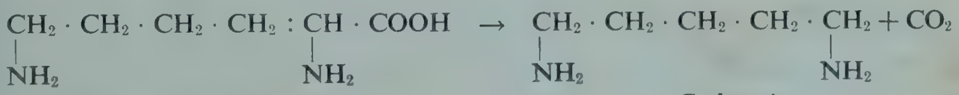
Direct Smears. Sometimes direct microscopic examination of smears made from the food, solid or liquid, will give much valuable qualitative information, as, for example, the detection of streptococci and pus cells in milk. The smear method also provides roughly quantitative data. It is useful for surface scrapings of meat, fermented products, etc. If the contents of a can shows only sporeforming rods in smears, it is probable that spoilage is due to spores of one or two species which resisted processing. If a mixture of many forms, including nonsporeformers, is seen, it indicates that the processing was inadequate or that the container has gross leaks or that the food was very dirty. Coupled with a plate count to find out how many of the organisms seen in the smear are alive, a very shrewd estimate of the history of the food in the can can be made. The direct microscopic method is used to examine products like catsup for mold filaments in order to detect excessive use of spoiled fruit in preparation of the food.

Opening Containers. In examining foods packed in containers, as canned goods, oysters in shells, etc., detailed directions are often given for the process of opening or piercing the container. It is obvious that if the surface of the container is not thoroughly cleaned and sterilized before piercing, organisms will be introduced from the exterior at the moment of opening into what might otherwise be a sterile product. If there is a removable cover on the container every effort should be made to remove dirt and dust, particularly around the edges close to the mouth of the container, as it is here that contamination is most likely to be introduced. Thorough scrubbing with a brush, application of disinfectants and washing with a fast, fine stream of hot sterile water are useful. The wash water should be *completely* removed and the surfaces dried before opening the vessel. The contents of cans can best be removed through a small opening sterilized by the application for fifteen seconds of an intense needle-blast flame, or by thorough chemical disinfection, followed by washing and drying. Sterile openers should be used. In heating cans, internal pressure may be generated, especially if the can contains gas from spoilage. As there is danger from explosion, care should be exercised in such work.

Specific Causes of Spoilage or "Diseases" of Foods. Sometimes conditions in food processes become especially favorable to the luxuriant growth of a single species of organism so that it dominates the flora of a given food and may produce some striking alteration of taste, odor, pigment, sliminess, or the like. A good example is "ropy bread" due to the use of flour heavily contaminated with spores of *B. mesentericus*, a slime-producer, the spores of which resist baking temperature and then germinate in the moist bread during storage. Canned oysters sometimes become contaminated with a red torula in a dirty shucking house in which growth of this red species has gotten well distributed. The oysters and fluid in the cans are covered with the red growth and not salable although no marked change in odor or taste is produced and the red torula is presumably harmless when eaten. Butter sometimes acquires a rancid, fishy or oily taste or odor because of the growth in it of large numbers of some particular organism which had grown richly. Perhaps a dirty churn may have heavily seeded the whole lot of butter. Sometimes a red, blue or yellow or other color is imparted to milk, fish, etc., by heavy growth in it of a *Serratia* species, a *Pseudomonas* or a *Flavobacterium.* Various spottings, ammoniacal decompositions and peculiar types of fermentation are produced under special circumstances and by specific organisms.

Usually the control of such specific types of spoilage is not too difficult, once the organism is isolated and its growth peculiarities and source studied. Contamination with mixtures of organisms found in soil, dust, decks of fishing boats, floors of slaughter houses, canneries, dairies, etc., is really the most difficult to control since it cannot be dealt with by means of a single specific measure designed especially to eliminate one particular source of contamination—as a single piece of machinery or container—but must be eradicated by measures of general cleanliness which may involve considerable expensive manpower, steam and hot water and general disinfectants on a very wide scale and against continuous reintroduction of dirt.

Ptomaine Poisoning. The word ptomaine is derived from the Greek ptoma, a dead body. Ptomaines were formerly believed to be formed in cadavers by intrinsic mechanisms and to be deadly poisonous. Chemically ptomaines are organic bases related to the amines or substituted ammonias. They are formed during advanced decomposition of proteins by proteolytic microorganisms and naturally occur in badly decomposed protein-containing products. They probably result from the decarboxylation of certain amino acids, especially lysine, which yields cadaverine; and arginine, which results in the formation of putrescine. For example:



$$\underset{\underset{NH_2}{|}}{CH_2} \cdot CH_2 \cdot CH_2 \cdot CH_2 : \underset{\underset{NH_2}{|}}{CH} \cdot COOH \rightarrow \underset{\underset{NH_2}{|}}{CH_2} \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot \underset{\underset{NH_2}{|}}{CH_2} + CO_2$$

Lysine *Cadaverine*

In such decomposed products as Limburger cheese some of them may be present in considerable amount and add desirable aroma and flavor. Only a few are violently toxic. A large amount of rotten food would have to be ingested to acquire an effective dose of any ptomaine so that, under ordinary conditions of diet, ptomaine poisoning does not occur. Practically all cases of so-called ptomaine poisoning are infections or intoxications due to bacteria of the *Salmonella* or *Staphylococcus* groups.

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Chapter 39

MICROBIOLOGY AND INDUSTRY

"The microorganisms appear to have gone into business. Imperceptibly their myriads have crept into industry, where they are nourished and pampered for the work that they do."* The effects of the growths of certain microorganisms are being made use of more and more widely in the industries and "germs" are more and more being regarded as the servants of man. As seen in an earlier chapter of this book, every living cell may be likened to a tiny chemical factory which forms, by means of its various enzymic and metabolic activities, varied and often extremely valuable substances or changes.

For many years it has been known that microorganisms play an important role in the manufacture of a number of substances used commercially. Some of these biological processes have been very extensively investigated by expert microbiologists, and in a number of processes certain specific organisms have been discovered as the active and essential agents. This is particularly true in the manufacture of commercial solvents (alcohols of various kinds, acetic and other organic acids, acetone and glycerin). In a number of processes, however, final data on the exact role of definite species of microorganisms have not yet been obtained. The fermentation of coffee and cocoa are examples of the empirical type of bacteriology in industry. The field of industrial microbiology is an open one and offers excellent opportunities for the microbiologist. The tremendous importance and value of industries based on the activities of microorganisms is shown in an interesting review of the fermentation industry during World War II. It is to be remembered that fermentation is only one branch of industrial microbiology.[1]

FACTORS OF IMPORTANCE IN DEVELOPING INDUSTRIAL PROCESSES

In arranging or studying an industrial process based upon the action of microorganisms a great many details must be given consideration.[2] The more important of these may be grouped, roughly, in four categories.

1. Purity of Cultures. It must be ascertained whether absolutely *pure* cultures *must* be used, or whether the mere *predominance* of one organism is sufficient. This may be a deciding factor, as the cost of preparing and maintaining pure cultures and sterile apparatus, etc., throughout a process may be excessive. For purposes of discussion let us assume a pure culture to be requisite.

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* Flint, from Smyth and Obold, "Industrial Bacteriology," Williams and Wilkins.

The organism must be able to grow well in the medium to be used and under the conditions of the process. This will entail very exact studies of *optimum* conditions of aerobiosis, temperature, nutrition and *p*H, and an appropriate adjustment of the process and apparatus to provide those conditions. The organism must be one which will evolve the desired end-products or produce the desired result in the medium furnished, in amounts or degree sufficient to yield a profit. Some firms have "pet" strains of microorganisms for producing certain products, such as butyl alcohol, or itaconic acid,[3] which they have "developed" (selected variants) for these purposes. Within the last few years it has been found possible to induce mutations in microorganisms by ultraviolet radiation (see Chapter 8), and by this means new and valuable types of industrial microorganisms are developed. The "breeding" of yeasts and molds for similar purposes is analogous to breeding of cattle for beef or milk, etc. (Chapters 21, 22).

2. Medium or Raw Material. A second factor to be considered is the subtrate or medium. It should support luxuriant growth of the organism to be used. Further, it must be available constantly and at costs compatible with profit. Expensive handling machinery may be needed for some substrates.

An important item is the possible necessity of a preliminary treatment such as liming of very acid yeast "slops," distillery wastes, molasses and whey. Some substrates, such as sawdust or fiber, may need preliminary "digestion" with hot acid or alkali to hydrolyze them to fermentable substances. This all adds to the expense and time.

3. Nature of the Process. A third consideration, the details of which arise from the requirements of the first two, is the process itself. It is obvious enough that the more complicated and exacting the system of cultural details and preliminary heatings, dilutings and digestions, as well as the type of machinery ("cracking" stills, tanks, pumps) to handle the end- and by-products and the final wastes, the greater the cost and therefore the less the commercial practicability of any process. Any time-consuming "aging" or "ripening" processes eat into the credit side of the ledger. Sometimes very desirable end- or by-products may be found in commercial fermentations, yet the cost of their recovery may be prohibitive.

4. Preliminary Experimentation. The fourth matter to be considered in industrial microbiology is preliminary trial and investigation. The microbiologist working with 10 ml. test-tube cultures may find many valuable things. When attempts are made to reproduce the test-tube experiments on a 100,000-gallon factory scale, however, the laboratory discoveries often fail to yield the promised result. Any process developed in the experimental laboratory must next prove its worth in the factory. All may depend on such a seemingly far-removed detail as international relations. These may affect the cost of importation of some raw product essential to the process under investigation. Then the industrialist turns to home resources, goes to Washington, or *employs a resourceful microbiologist!*

The whole matter is a complex of microbiology, chemistry, engineering and economics and cannot be more fully discussed here. Many of the chemical and microbiological processes in use at present are patented and secret, and specific strains of bacteria, yeasts and molds, which are zealously guarded, are often carefully developed in the laboratories of manufacturing concerns. We shall, therefore, present only general principles and point out the possibilities for research and advancement in industrial microbiology, leaving the student, if he is interested, to consult original papers for greater detail.

INDUSTRIAL PROCESSES

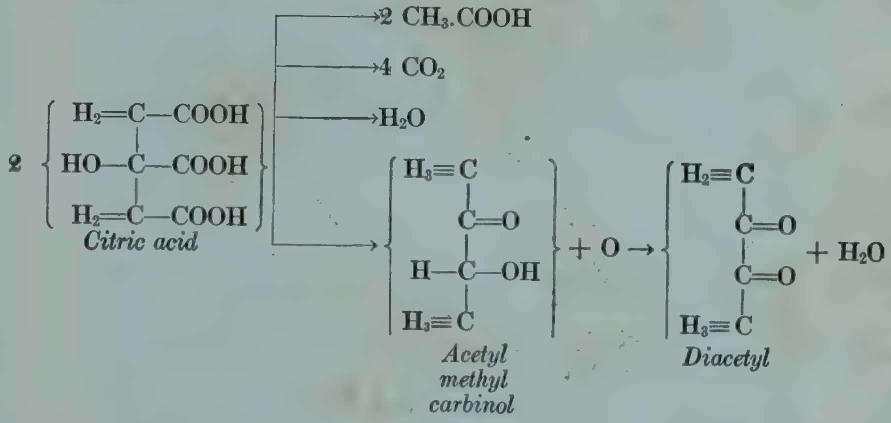
In commerce fermentation, oxidation, proteolysis, lipolysis, synthesis and other activities of microorganisms are employed. The products differ, depending upon the kinds of microorganisms involved and the substance which they are required to alter or grow upon, and the conditions of growth during the process. One industry of very ancient origin, but in which modern bacteriology has come to play an important part, is cheese manufacture. Both fermentation and proteolysis, as well as lipolysis, are important in this process.

CHEESE AND OTHER DAIRY PRODUCTS

The use of members of the genus *Lactobacillus* and of *Streptococcus lactis,* as well as of yeasts, in preparing fermented milk beverages has been described (page 639). Pure cultures of *Lactobacillus* (*L. helveticus*) are also widely used as starters for various dairy products which depend on lactose fermentation. It has been pointed out also that some butter is made by churning cream which has been soured by means of cultures of *Str. lactis, Str. cremoris* and related organisms. These form pleasantly flavored volatile products during their fermentation of the lactose or milk sugar. Their rapid growth in the preliminary stages of butter-and-cheese-making produces an acidity which inhibits development of undesirable bacteria such as the coli-aerogenes group. Cultures of the desirable organisms are obtainable commercially for the purpose of inducing favorable souring, and are known as "starters."

Butter. *Streptococcus lactis* and *Str. cremoris,* commonly occurring in milk, are not alone responsible for the desirable flavors in butter.

Closely related species capable of attacking citric acid under acid conditions, *Leuconostoc citrovorum* and *Leuconostoc dextranicum* (also classed as *Str. citrovorus* and *Str. paracitrovorus,* respectively) are of great importance. The exact relationships of these species is not clear but it seems likely that some *Leuconostoc* may represent encapsulated variants of *Str. citrovorus* and similar species. They produce diacetyl (see below) which has a pleasant, "buttery" aroma. The addition of a little (0.1 per cent) citric acid to cream or milk for butter aids in the production of these aromas, while the necessary acidity for satisfactory flavor production is produced by *Str. lactis, et al.* Commercial or home butter starters should contain these citric acid-fermenting strains in large numbers.



Sterilized milk is used as a medium for maintaining or shipping transfers from prepared mixtures of these organisms. Much experience is required to maintain the mixed cultures in such a condition that they will give the best results. Apparently the organisms vary considerably.

There are many molds and bacteria which cause spoilage of butter. Development of undesirable flavors in butter, especially rancidity, is due in great part to the formation of butyric acid as a result of lipolysis[4] especially by species of *Aspergillus* and other molds and streptococci related to *Str. liquefaciens.*

Cheese. The manufacture of cheese is one of the industries of antiquity. The bacteriology of the processes, however, is still not completely known but is understood to a degree sufficient for the desired types of cheese to be made.

Three general types of cheese may be mentioned, (*a*) soft, acid-curd or cottage type cheese and cream cheese (these are eaten in a fresh or unripened state); (*b*) hard- or rennet-curd cheese, including American, Roquefort, Cheddar, and Swiss or Emmenthal (these are "ripened" by the growth in them of bacteria or molds or both, which do not cause extensive proteolysis); and (*c*) soft or semisoft rennet-curd cheese, of which Camembert, Limburger and Liederkranz are types. These are ripened by more or less proteolytic organisms which soften the curd. The hardness of cheese depends to some extent on moisture and fat content as well as on heating and acidity of the curd, salting, and conditions of storage. There is some disagreement as to the classification of cheeses. A list of cheeses is given in Table XV.

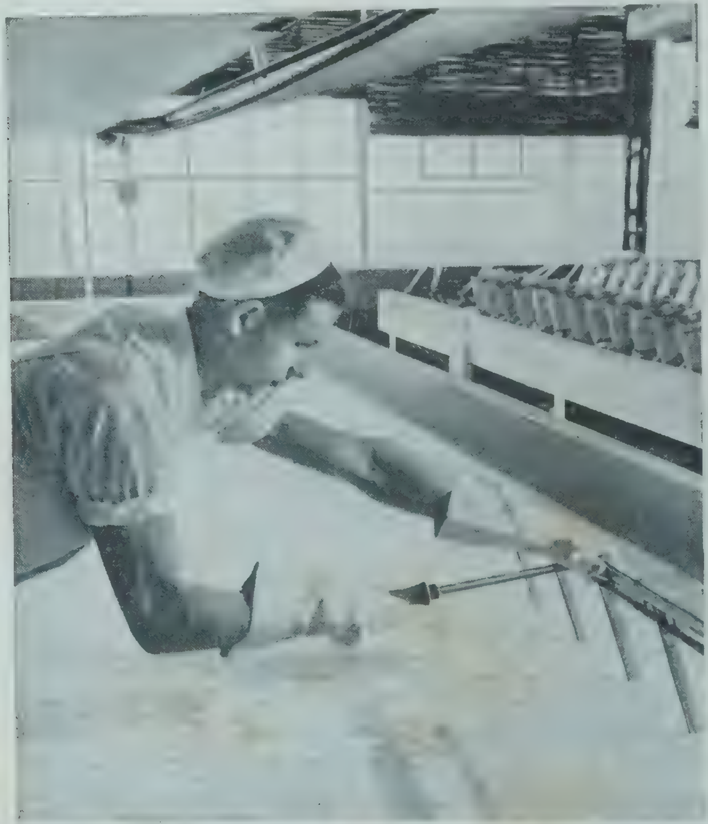


Fig. 314. Liquid milk becomes "curd" and "whey." After "starter" and rennet are added, the milk forms into a soft curd much like "junket." When it has reached the correct firmness, it is cut into small cubes (¼ inch) by special wire knives. The whey is slowly expelled from these cubes as the curd and whey are heated, while agitated by revolving paddles. The picture shows the cutting operation. (Courtesy of Kraft-Phenix Cheese Corporation.)

Soft, Acid-curd Cheese. In cheeses of this type, represented by "cottage cheese," the bacteria normally present in the milk are allowed to ferment the lactose, the lactic acid which is thus formed coagulating the casein which is then strained out, drained and sold. Rennet may be added to hasten the coagulation and make the curd firmer. Salt may be added to taste after draining the curd or during the curing process. *Streptococcus lactis* is the predominating organism, although others may be added in pure culture as starters. Certain yeastlike plants (*Oidium lactis*) also aid. Proteolytic organisms like *Str. liquefaciens* are responsible for undesirable bitter flavors and early spoilage. It is important to use fresh, clean milk to avoid gas production by *Aerobacter*, etc., anaerobes, and putrefaction by *Pseudomonas, Bacillus*, etc. Hot pressed, flavored cream may be made into cream cheese.[5]

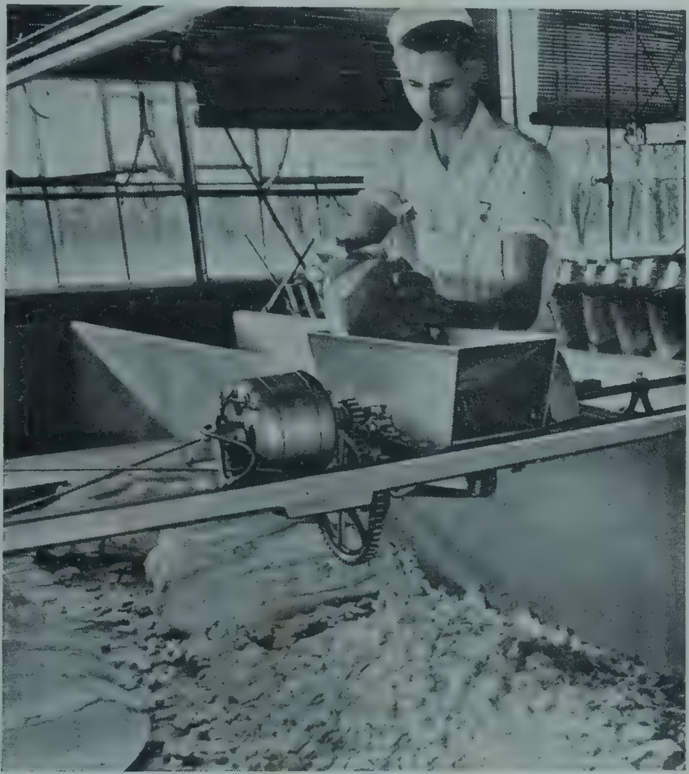


Fig. 315. The curd is milled, salted. When cheddaring is complete (judged by the firmness of the curd, its texture and acidity) the curd is milled (cut into small pieces) in preparation for salting and pressing. About 1½ pounds of salt are used to 100 pounds of curd. After salting, the curd is placed in cheesecloth or cellophane lined "hoops" of the desired size and shape for pressing. (Courtesy of Kraft-Phenix Cheese Corporation.)

Hard-curd Cheese. In making hard- or rennet-curd cheeses, such as Cheddar cheese, the milk is usually first allowed to become slightly acid through normal fermentation or souring. Sometimes cultures of *Str. lactis* or *Leuconostoc dextranicum* are added to hasten this and improve flavor.[6] Rennet is then added to make an elastic, rubbery curd which is later chopped (Figs. 314 and 315) into pieces about one inch in diameter and heated. After heating to about 95° F. the curd becomes firmer and whey separates and is drained off and may be used for stock feed. The clumped masses of firm curd are chopped again, and piled up to press out whey. This is called "cheddaring" in Cheddar* cheese making. The curd is again milled, salted, drained, and pressed in hoops to ripen. Ripening goes on at about 55° F.

During the curing process various microorganisms, the varieties depending on the kind of cheese (*e.g., Str. lactis, Str. cremoris,* lactobacilli, *Oidium lactis, Aspergillus* and *Penicillium*), are allowed to continue a slow fermentative, lipolytic, and putrefactive action, the products of these processes yielding the substances responsible for the characteristic flavors, textures and aromas of various cheeses. Prominent among these are lactic, butyric and acetic acids, ammonia and various amines, derived from the decomposition of the lactose, the fat and the proteins, as well as various esters such as those which give flavors to ripe fruit juices. In addition, since many of these organisms synthesize vitamins, especially nicotinic acid, and vitamins of the B complex, the nutritive value of the ripened cheeses is increased.

TABLE XV

CHEESES[7]

Soft cheeses:	
Cottage	Not ripened
Cream	
Camembert	Ripened by molds
Brie	
Limburger	Ripened by bacteria
Liederkranz	
Hard cheeses:	
Gorgonzola	Ripened by molds
Roquefort	
Emmenthal (Swiss)	Ripened by gas-forming bacteria (holes)
Parmesan	
Edam	Ripened by bacteria (no holes)
Cheddar	

The *Escherichia, Aerobacter* and *Clostridium* are undesirable in cheese making since they produce gassy cheeses and "off" flavors; they may be especially active in the early stages. It has already been pointed out that *Propionibacterium* are active in the ripening and flavor of Swiss cheese. The "eyes" in Swiss cheese are due to the production of carbon dioxide by the bacteria, while flavors are due in part to the formation of glycerol, propionic and succinic acids by these organisms.

Much depends on the way the cheese is made.[8, 9, 10, 11, 12] Swiss cheese (Fig. 316) is heated to about 55° C. early in the process to prevent overgrowth of the streptococci and permit growth of the thermophilic lactobacilli (*L. thermophilus, L. lactis,* etc.) which give a certain desired texture and flavor. These subside after a time and propionibacteria are then favored by incubation at lower temperature.

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* Cheddar is the name of a town in England famous for this type of cheese making.

Roquefort cheese contains as high as 4 per cent salt and relatively little moisture. This prevents growth of most bacteria, as does also the low ripening temperature of 9° C., although *Str. lactis* may continue in large numbers for several days after the initial souring. A species of mold (*Penicillium roqueforti*), however, is introduced by the admixture of spores with the curd before it is put into the hoops for ripening. These grow in the interior producing the "sharp" flavor so characteristic of this type of cheese (Fig. 318). As the mold is aerobic, perforations are made in the cheese to aerate the interior.

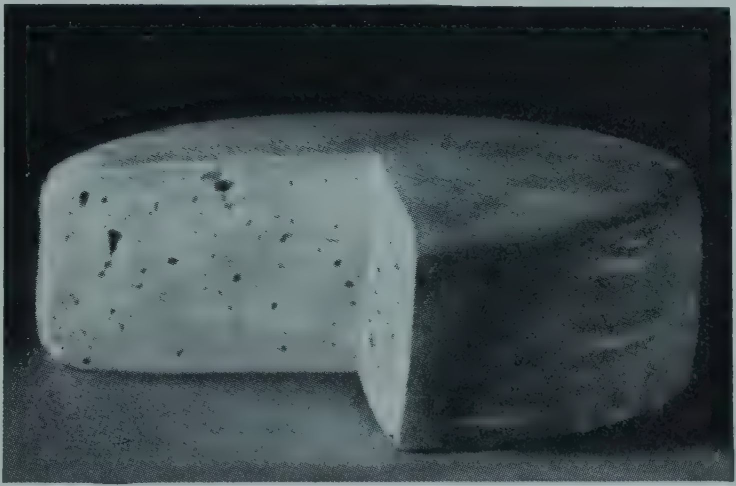

Fig. 316. Swiss type cheese, showing holes due to gas produced by the bacteria in the cheese before it hardened. (Courtesy Hansen's Laboratory, Inc.)

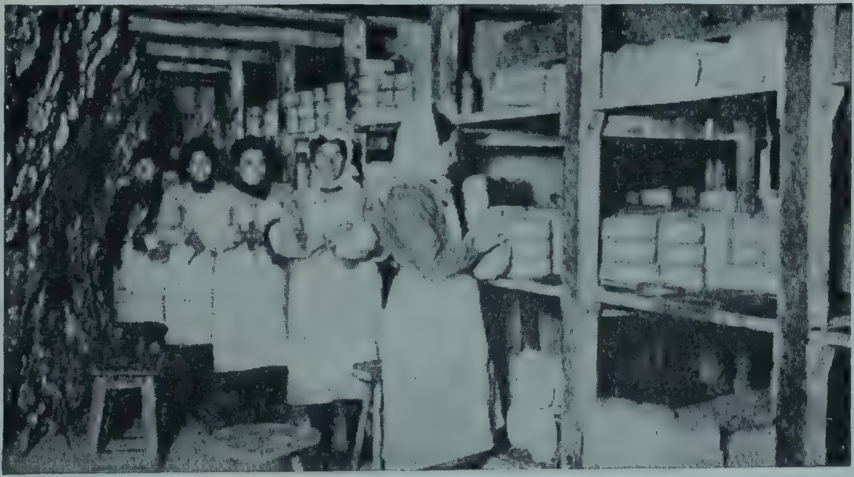

Fig. 317. Cave in Roquefort, France, in which conditions are particularly favorable for the ripening of Roquefort cheese. See also Figure 319.

The flavor results from the production of caproic, caprylic and capric acids from hydrolysis of the butter fat, as well as from the delectable volatile products of casein and lactose decomposition, some of which have been mentioned above. The development of proper flavors and texture depends in great part on suitable conditions of temperature and humidity of the atmosphere. In France the air in certain caves is especially favorable. In the United States air-conditioning of the ripening rooms is resorted to (see Figs. 317 and 319).

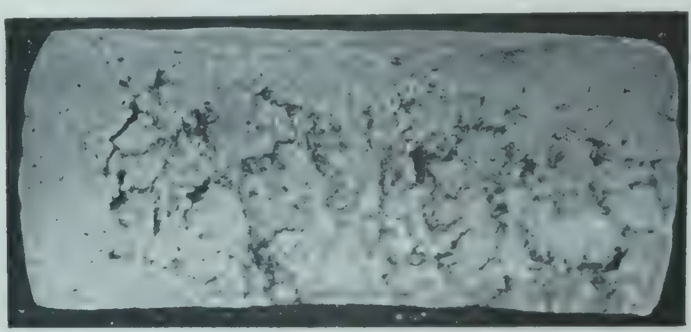

Fig. 318. Roquefort cheese, showing the mold (*Penicillium roqueforti*) penetrating the interior of the mass. (Reprinted by permission from B. W. Hammer's "Dairy Bacteriology," John Wiley & Sons, Inc., publishers.)

Fig. 319. Camembert cheeses ripening in a room which is carefully "air-condi tioned." (Courtesy of Kraft-Phenix Cheese Corporation.)

In cheeses like Camembert the mold (*Penicillium camemberti*), being more strictly aerobic, grows on the surface, the liquefaction characteristic of this cheese proceeding from without inward. Probably *Oidium lactis* also plays an important role in the ripening (Fig. 319). Camembert cheese contains more water and less salt than Roquefort and is therefore softer to begin with.

Home Cheese-making. It is not difficult to make Cheddar-type cheese at home. Two gallons of sweet milk (cow's, goat's, etc.) are warmed in an enameled vessel to 86° F. Yellow coloring (available at farm-supply houses) is added to suit. Two or three tablets of rennet or "Junket" tablets (available at grocery stores) dissolved in an ounce of cold water are stirred in. The vessel is kept at 85° to 90° F. for about thirty minutes, or until a firm "dry" (no free fluid) curd is formed. The curd is then cut by passing a long, narrow, knife blade through it until cubes about ½ inch thick are formed.

The contents of the vessel are then brought to about 95° F. by gentle warmth. The contents are *gently* moved about with a wooden spoon to equalize the temperature and separate the cubes of curd, which shrink. After about 40 minutes the contents of the vessel are emptied into a cloth bag (a clean 50-lb. salt bag or a "feed" bag will serve) and hung to drain. After three or four hours the whey has drained off. Lay 3 or 4 thicknesses of cheesecloth on a board, such as a pastry board. Make a circular bandage of cheese-cloth, 3 or 4 thicknesses through, 4 inches wide and about 8 inches in diameter. Set this on edge on the cloth on the board. Empty the drained curds into the bandage and draw it up so that a firm, solid, disc of cheese is formed, level across the top. Cover with a layer of cheese-cloth folded double.

On top of the disc place another board and on this place a weight, such as a flat iron or a brick. After about 8 hours, carefully turn the disc over and replace the weight. After another ten to fifteen hours, remove the covering and keep the cheese in a cool, moist place, but do not freeze. Turn the disc over 2 or 3 times each day. A rind will gradually form, after which ½ ounce of salt may be rubbed over the cheese on two successive days. The turning and rubbing may be continued for several days, but no more salt is needed. The cheese is ready for consumption after four or five weeks.

VINEGAR AND ACETIC ACID. THE ACETOBACTER

Acetic acid is almost entirely responsible for the sour taste of vinegar. Indeed, a slightly sweetened, 3 to 5 per cent aqueous solution of chemically pure acetic acid makes a passable substitute for vinegar. Commercial vinegar contains 4 per cent acid. The acid of naturally soured vinegar is derived from alcohol by the action of living organisms which are included in the family Pseudomonadaceae (genus *Acetobacter*). Pleasant flavors of natural vinegar are given by traces of various esters like ethyl acetate, and by alcohol, sugars, glycerin, volatile oils, etc., in small amounts. The *Acetobacter* thrive on the surface of wine, "hard" cider, beer, or any nutrient liquor containing alcohol. Preliminary fermentation to produce the necessary alcohol is often carried out by means of *Saccharomyces cerevisiae* (brewers' yeast). However it may occur spontaneously, as when cider turns "hard." The *Acetobacter* later form a thick scum or pellicle, oxidizing the alcohol to acetic acid as a source of energy and utilizing other substances present as sources of food. They often ruin wine during the home manufacture of this beverage. The pellicle tends to become slimy with age (mucoid variation) and then constitutes the "mother of vinegar."

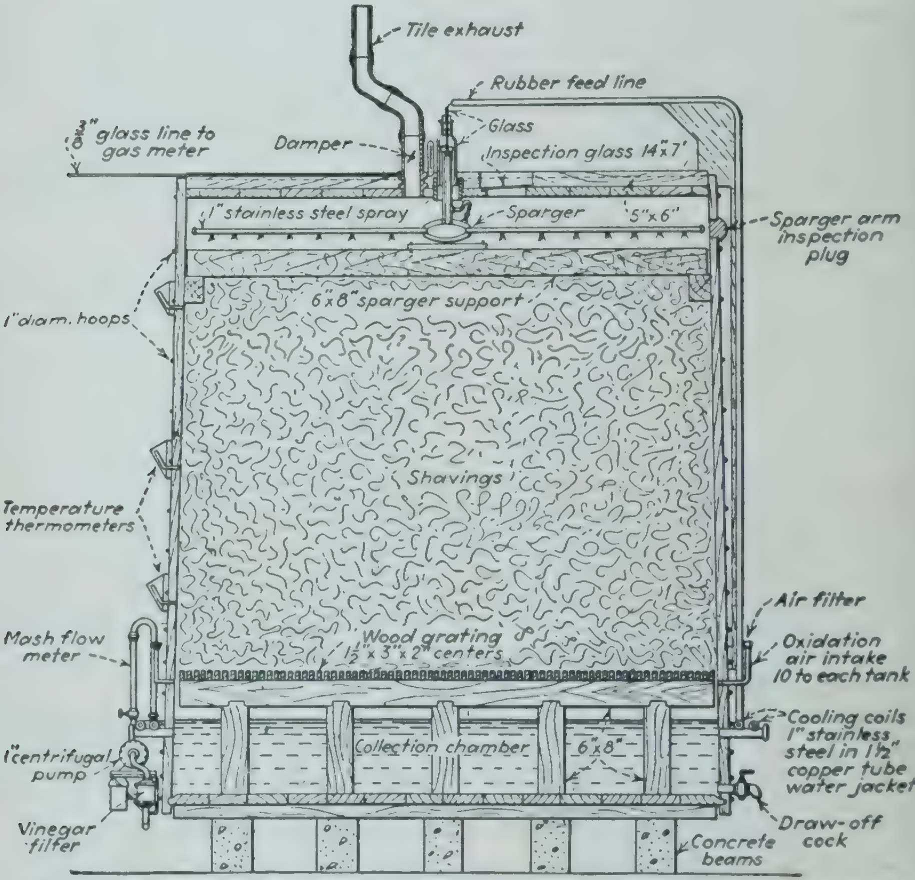
Tile exhaust
Rubber feed line
3/8" glass line to gas meter
Damper
Glass
Inspection glass 14"x7'
1" stainless steel spray
Sparger
5"x6"
Sparger arm inspection plug
1" diam. hoops
6"x8" sparger support
Shavings
Temperature thermometers
Air filter
Mash flow meter
Wood grating 1½"x3"x2" centers
Oxidation air intake 10 to each tank
Cooling coils 1" stainless steel in 1½" copper tube water jacket
1" centrifugal pump
Collection chamber
6"x8"
Draw-off cock
Vinegar filter
Concrete beams

Fig. 320. Cross section of the Frings generator. The alcoholic liquor is sprayed over the shavings by the rotating stainless steel spray (sparger) near the top. Note the thermometers, cooling coils and air intakes. (Courtesy A. E. Hansen, Food Industries, vol. 7.)

Inasmuch as the change from alcohol to acetic acid is principally an aerobic oxidative process, it is evident that it will go on best when a large surface is exposed to the air for the growth of the *Acetobacter*, since they are strict aerobes. Therefore, in a commonly used commercial process, known as the "quick" or "generator" process for the production of acetic acid, alcoholic fluids are made to trickle in a thin stream through a tall cylinder filled with shavings of beechwood, wicker-work, coke or tile, previously inoculated with *Aceto-*

bacter. Air is made to circulate freely through the cylinder around the wet surfaces of the shavings. Heat is evolved and in large generators used in commercial work cooling devices must be resorted to. Due to the acidity of the product, wooden tanks and stainless steel or other resistant pipes are used. The acidity also eliminates much of the danger of contamination by extraneous organisms (Fig. 320).

The *Acetobacter* are rods about 0.5 microns by 0.8 microns, although species vary in size. Branching involution forms and large, swollen cells frequently occur. Some species are motile. A species of historical interest is *Acetobacter* (*Mycoderma*) *aceti,* originally used by Pasteur to demonstrate the biological nature of vinegar formation. Species important in commercial vinegar production are *A. schuezenbachi* and *A. orleanense*. In actual practice, several species of *Acetobacter* act jointly. The overall reactions probably are as follows:

$$\begin{cases} \underset{\textit{Alcohol}}{C_2H_5OH} + \tfrac{1}{2}O_2 = \underset{\textit{Acetaldehyde}}{CH_3CHO} + H_2O \\ CH_3CHO + \tfrac{1}{2}O_2 = \underset{\textit{Acetic acid}}{CH_3COOH} \end{cases}$$

Home Vinegar Manufacture.[13]—Recommendations for home vinegar manufacture illustrate the application of general principles to the slow or Orleans process. Apples are selected which are fully ripe, but sound and undecayed. Apples of high sugar content yield vinegar of high acid content, since it is the sugar which is eventually turned into acetic acid. *Red Astrachan, Tolman Sweet* and *Sweet Bough* are useful varieties. The cider is allowed to settle for two or three days and is pumped or siphoned into barrels (clean and scalded to kill microorganisms producing undesirable fermentations) until they are about three-quarters full. The barrels are laid on their sides with the bung-holes open (but screened) and up. A cake of yeast or a pure culture of selected wine yeast is emulsified in half a cupful of water and added to each barrel. This promotes alcoholic fermentation, which is complete in from three to six months, depending on temperatures. In commerce temperatures are carefully controlled. At the end of this time, the clear alcoholic liquor is drawn off. If the alcohol content is more than about 10 per cent, the fluid must be suitably diluted, as too much alcohol is unfavorable to *Acetobacter.*

A small amount of good quality natural vinegar or lactic acid is added to adjust the acid content so that it is unfavorable to undesirable bacteria. This may require 5 per cent by volume, or an acidity around *p*H 5. The use of vinegar for this purpose also accomplishes inoculation. As the oxidation of alcohol to vinegar is strictly aerobic, the bung-hole must be left open. A cotton plug is useful to keep out extraneous organisms. Up to this stage, the barrels have been filled only three quarters full, in order to allow a broad surface for the absorption of oxygen. But when acidification has reached completion, the barrels are filled, the bungs driven in and the product stored in a cool place to preserve the vinegar. Exclusion of oxygen is requisite now to prevent further oxidation of the acetic acid to carbon dioxide and water by aciduric microorganisms, such as yeasts. In commerce, pasteurization is sometimes used to stabilize the vinegar if it is not to be used at once.

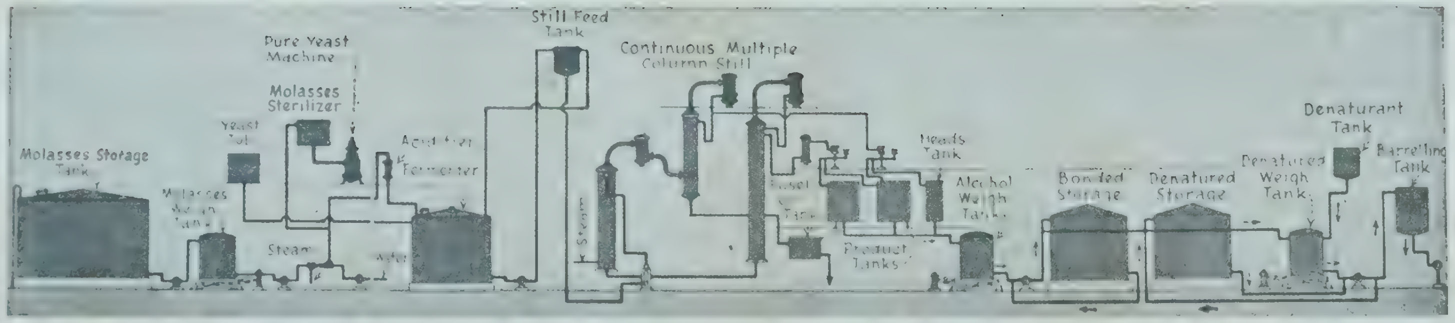
Molasses Storage Tank
Molasses Weigh Tank
Yeast Tub
Pure Yeast Machine
Molasses Sterilizer
Steam
Water
Acidifier
Fermenter
Still Feed Tank
Steam
Continuous Multiple Column Still
Fusel Tank
Product Tanks
Heads Tank
Alcohol Weigh Tank
Bonded Storage
Denatured Storage
Denatured Weigh Tank
Denaturant Tank
Barrelling Tank

Fig. 321. Arrangement of equipment in a modern plant for the production of ethyl alcohol by the fermentation of black strap molasses. (From R. R. Collins, The Lummus Co., Chemical & Metallurgical Engineering, vol. 41.)

ALCOHOL MANUFACTURE

Commercially, alcohol is derived from the fermentation of various carbohydrates, chiefly by yeasts. Starches and cellulose which may be hydrolyzed by means of enzymes or heat and acid to simpler carbohydrates which yeasts can ferment, may be used. Corncobs and sawdust may be so treated. A much used source of industrial alcohol in the United States is crude molasses. It is easily handled in pumps and tanks and is always available. It requires only to be diluted and the *p*H adjusted. Dilution is necessary to avoid inhibition of the yeast by osmotic pressure, and uneconomical utilization of the sugar. A concentration of about 12 per cent sugar is satisfactory. The *p*H is adjusted, usually with sulfuric acid, to around 4.5. This is favorable to the yeast and unfavorable to bacteria. Sometimes nutrients may be added, especially a source of nitrogen such as ammonium sulfate. Properly prepared carbohydrate "mashes" such as the above are sometimes freed from excess undesirable live bacteria by heat, but this is expensive.

The mash is inoculated with an aciduric and alcohol-resistant strain of yeast, the variety depending on the conditions under which the fermentation is to proceed and the exact end-products desired. The inoculum comes from a large tank of carefully maintained pure culture previously inoculated from a smaller seed tank and the latter from a flask or tube of culture (Figs. 321 and 322). The inoculum tank or "yeast machine" is aerated as this promotes rapid growth of yeast cells. The maintenance of purity of the inoculum is a responsibility of the bacteriologist, and woe betide him if some sporeformer or "wild" yeast gets in and ruins 100,000 gallons of "mash"!

Fermentation is allowed to continue in vats holding as much as 50,000 gallons for about forty-eight hours at a carefully controlled temperature of about 25° C. until the yeast stops growing due to the concentration of alcohol and other products (Figs. 323 and 324). Aeration may be used at first to promote rapid growth, but anaerobiosis is soon established to promote alcohol accumulation and prevent its oxidation to carbon dioxide and water. The temperature tends to rise but is kept down by streams of cool water sprayed on the fermentation tanks or by other means. Some yeasts can become habituated to growth until the alcohol concentration of the mash has reached as high as 16 per cent.

After fermentation the crude alcohol or "high wine," as it is called, is usually a mixture of ethyl alcohol and a small amount of glycerol with fusel oil. The last contains amyl, isoamyl, propyl, butyl and other alcohols with acetic, butyric and other acids, as well as various esters.

The high wine is driven off from the mash or "beer" by heat and further purified by fractional distillation.

The chemical reactions involved in the fermentation are very complex. The chief constituents of fusel oil are probably derived from amino acids in the mash. The large amounts of carbon dioxide evolved are purified and compressed in tanks or made into "dry ice." Part of this may be used for cooling the vats. The common intermediate substances, methyl glyoxal, pyruvic acid and acetaldehyde, take part in the series of reactions, and preliminary combination of the dextrose with phosphoric acid (phosphorylation) occurs.[2]

Production of Alcohol by Bacteria. Certain aerobic bacteria (*Bacillus acetoethylicus** or *Bacillus macerans*) are also capable of producing ethyl alcohol as well as the very valuable substance, acetone, from carbohydrate materials. Molasses, potatoes and other materials are used.

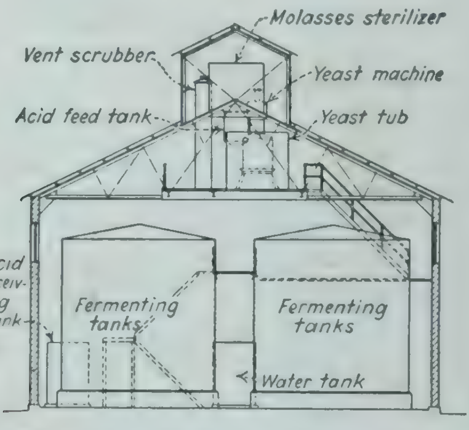

Fig. 322. Elevation of the fermenting room showing yeast equipment. (From R. R. Collins, The Lummus Co., Chemical & Metallurgical Engineering, vol. 41.)

Studies of the biochemical properties of highly fermentative bacteria of this kind may yield more species of even greater commercial value. *B. macerans* is weakly gram-positive, motile and grows well in ordinary laboratory media.

For alcohol production by these bacteria cellulose-containing wastes may be hydrolyzed by steam and sulfuric acid. Corn cobs and oat and peanut hulls have been successfully adapted to alcohol production by this means. As in other fermentative processes, conditions of growth and incubation must be made suitable to the organisms involved and productive of the end-products desired. For *B. macerans* nitrogenous materials must be added if not already present, and other foods which the deficiencies of different mashes may require. The process is an aerobic one. The *p*H for maximum alcohol production by this organism is around 7.0; a *p*H around 6.0 results in more acetone production. The *p*H must be maintained by a buffer such as calcium carbonate. A temperature of about 42° C. is suitable. Incubation periods of about six days are required.

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* *B. acetoethylicus* is also important in flax retting. It is probably identical with *B. macerans*. It produces the enzyme pectinase. The spore forming aerobes (genus *Bacillus*) to which *B. macerans* belongs, have already been discussed (page 499).

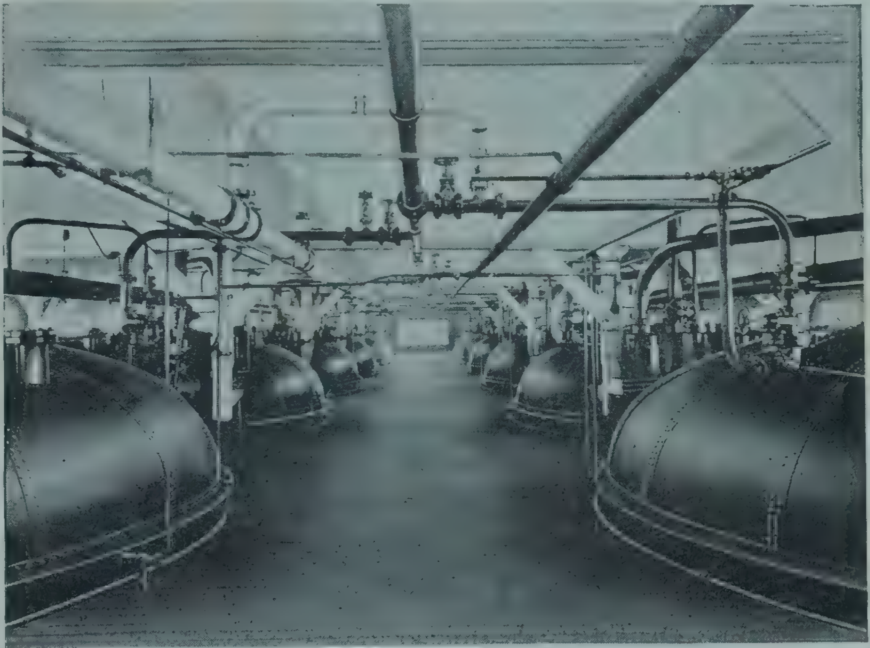

Fig. 323. Upper level of 50,000-gallon fermentation tanks. (Commercial Solvents Corp.)

The exact chemical processes involved are complex, but in general they resemble some phases of the fermentation of glucose by *E. coli.* The overall reaction appears to be

$$\underset{\textit{Starch}}{2C_6H_{10}O_5} + 3H_2O \rightarrow \underset{\substack{\textit{Ethyl}\\\textit{alcohol}}}{2C_2H_5OH} + \underset{\textit{Acetone}}{CH_3CO{\cdot}CH_3} + 5CO_2 + 3H_2$$

Certain by-products like acetic and formic acids which appear in the high wine may be derived from nutrient substances in the mash, other than carbohydrates, such as amino acids.

Use of Raw Cellulose for Alcohol Production. A method of producing alcohol from raw cellulose by bacterial fermentation has been sought for some time, and with some success as several cellulose-to-alcohol fermenting bacteria have been discovered. For example, *Clostridium dissolvens* (*B. cellulosæ-dissolvens* of Khouvine)[14] attacks cellulose specifically and will not grow in a medium which does not contain it. Veldhuis, Christensen and Fulmer[15] used a mixture of thermophilic, cellulose-splitting organisms common in horse manure.

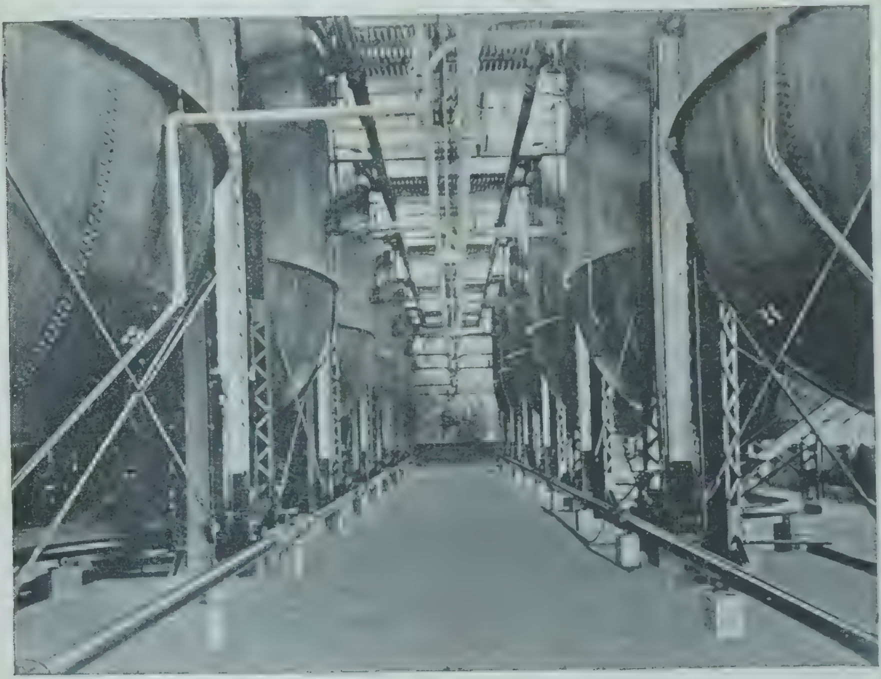

Fig. 324. Lower level of 50,000 gallon fermentation tanks. (Commercial Solvents Corp.)

In developing the fermentative combination of organisms, manure is introduced into a medium of the following composition:

Cellulose (pulped paper)	3.0	gm.
NH_4Cl	0.25	gm.
K_2HPO_4	0.25	gm.
$CaCO_3$	4.0	gm.
Tap water	100.0	ml.

The *p*H should be about 7.8.

The mixture is held anaerobically at 55° to 60° C. The culture is transferred to new medium every six days. This mixed culture ferments about 99 per cent of the cellulose at 60° C. Along with the alcohol there appear carbon dioxide, hydrogen, methane, and acetic, butyric and other volatile acids. This process has great possibilities, as the ratio of the various products may be varied by providing different media, *p*H, temperature and other factors, and because raw cellulose is a very cheap source of alcohol. It is especially noteworthy that pure cultures of the individual species of bacteria involved in the cellulose fermentation do not act nearly as efficiently as the mixture of bacteria normally present in the manure. Further researches along these lines, in order to develop more of such processes into profitable factory procedures, are desirable.

A pure culture process of this type may be based on a newly described species of *Clostridium* (see Fig. 325). Still another organism, a thermophil obtained from Finland, produces acetic, butyric and lactic acids from acid-hydrolyzed wood.[15a]

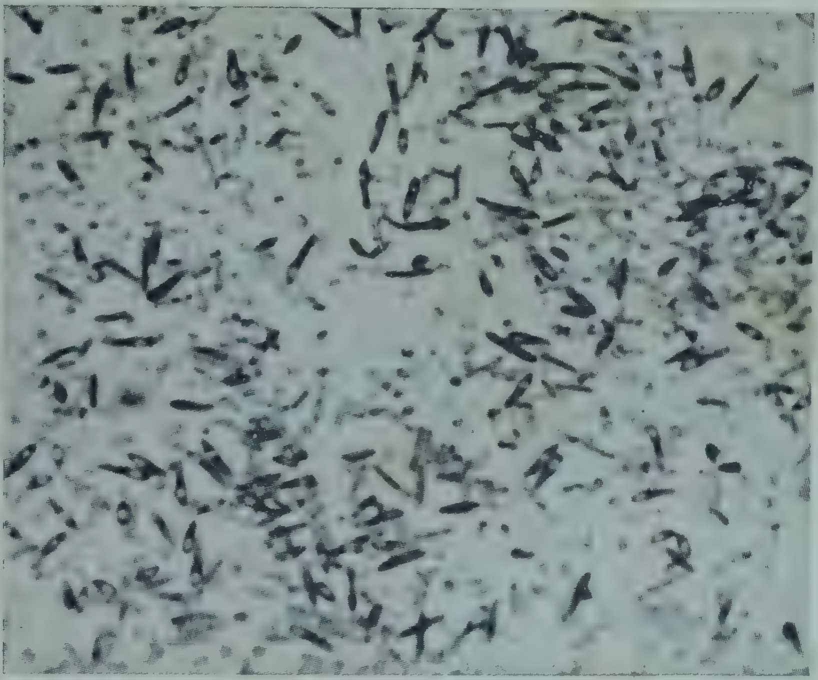

Fig. 325. *Clostridium lactoacetophilum* strain 3. Forty-eight-hour growth in medium 1 with 0.1 per cent agar. Free spores are visible in the background. × 1,000. (Bhat, J. V., and Barker, H. A., in J. Bact., vol. 54.)

Wood as a Source of Alcohol and Food. During the recent war the utilization of wood by-products ("slabs," sawdust, chips, stumps, etc.), was developed by the U. S. Department of Agriculture. In the Madison Wood Sugar Process the finely chopped wood (cellulose, with lignin, etc.), is treated by allowing 0.6 per cent sulfuric acid to flow slowly over it at a temperature of 150 to 180° C. The resulting liquid contains a variety of fermentable sugars, including glucose, galactose, mannose, xylose and arabinose. The liquid is brought to a *p*H of about 5 with lime and put under steam pressure. It is then filtered to remove $CaSO_4$ and other insoluble materials.

When cool, nitrogen is added in the form of urea and $(NH_4)_2SO_4$, the *p*H is adjusted to about 5.0 and the liquor is inoculated with

Saccharomyces cerevisiae. Fermentation is rapid, ranging from five or six to seventy-two hours, depending on the amount of inoculum used. Ethyl alcohol is produced in good concentrations and is of high quality.

In addition to the valuable products of fermentation, large quantities of yeasts are obtained. The yeast is valuable as stock feed and also for human consumption.

After distillation of the volatile products of fermentation the remaining "slops" or "still waste" contains nutrients which are used as a medium for further cultivation of yeasts which are valuable as food. The reaction is readjusted to around 4.5, unless the still waste already is at about the right acidity. More urea and $(NH_4)_2SO_4$ are added and the fluid is continuously aerated.[16, 17, 18, 19] Yields of up to 50 per cent the total reducing sugar consumed, in terms of dry torula yeast, are obtainable. These are fed to stock and so turned into meat and dairy products. Surely the transformation of a knotty old pine slab into a pork chop or a fried egg is modern magic!

Distilled Beverage Industries. The manufacture of alcoholic beverages is a time-honored occupation, reference to wine being found in Genesis, when Noah used it and became "drunken" (Gen. 9:21). In Chapter 1 some details are given concerning the part which Pasteur played as the first great industrial microbiologist in developing methods for the fermentation industries and solving their difficulties resulting from contamination of the "mashes." Today the production of alcohol for manufacturing and beverage purposes by fermentation and distillation is a very valuable and well developed industry. In principle the production of alcoholic distilled beverages is similar to the production of industrial ethyl alcohol. Refinements are introduced with respect to flavor, aroma, color, sanitation, etc., which are not necessary in the making of industrial alcohol.

Carefully selected grain is stored and ground as needed. Rye whiskey is prepared from mash containing at least 51 per cent rye; bourbon, at least 51 per cent corn. A "spirits mash" consists of 88.5 per cent corn, 1.75 per cent rye, and 9.75 per cent barley. The grain, mixed with water, is autoclaved, cooled, diluted, and 1 per cent barley malt is added. The malt, containing amylolytic enzymes, hydrolyzes the starch. The "mashing" or hydrolysis, proceeds in a special tank, at around 65° C., for about thirty minutes. The mash is then pumped to the fermentation tanks. Here it is heavily inoculated with selected yeast which has been cultivated in a mash previously made somewhat acid (*p*H 4.0) with lactobacilli as follows:

The yeast culture medium consists of mash similar to the main mash, but more concentrated. It is first hydrolyzed with maltase, inoculated with lactobacilli, and allowed to ferment till the *p*H reaches about 3.9. It is then heated to around 82° C. to stop the fermentation and kill the lactobacilli, cooled to 24° C. and inoculated with the yeast. The original sugar content of around 24 per cent is finally reduced to about 12 per cent by the yeast and bacilli.

As the main mash enters the fermenter, the yeast mash mixes with it. Water is added so that the final mash concentration is 36 gallons of mash per bushel of grain. The *p*H is adjusted by means of sulfuric acid, or acid residue from previous distillation, to about 4.9 at which point the remaining malt in the mash acts on the dextrins, etc. Contamination is also discouraged by this degree of acidity. Fermentation is complete in about seventy-two hours, as in industrial alcohol production.

In the distillation of alcoholic beverages, the fermented mash ("beer") is first distilled *in vacuo*. Aldehyde, esters and fusel oil are then separated from the "high wine" by further vacuum distillation. The purified product is then concentrated to about 95 per cent alcohol and further purified by fractional distillation in vacuo. The remaining details of distillation, blending, etc., do not involve microbiological principles.

The microbiologist maintains the yeast and *Lactobacillus* cultures; arranges precautions against contamination of the yeast culture vats, fermenters, etc.; makes frequent cultural checks on the various mashes; and examines the bacteria in the air, grain, water supplies, etc. Special investigations and "trouble shooting" add to his work.

BACTERIA AND SYNTHETIC RUBBER

The value of specialized research in this field is shown by the development by Liebemann et al.,[20] in 1943, of a variety of organism producing commercially feasible amounts of 2.3-butylene glycol or *butanediol* (see page 300) used in the manufacture of synthetic rubber. This glycol is formed directly during the fermentation of cornstarch mash. The butanediol is esterified and then fractionally distilled. Combined with styrene from petroleum it forms the basis of the synthetic gum. The Ford strain of *Bacillus subtilis* also produces 2.3-butanediol as well as glycerol.[21] Glycerol fermentation on a practical scale has been described by Fulmer, Vander Kofler and Hickey, who used a yeast, modifying its usual metabolic processes by adding sodium sulfite to the medium, and adjusting the *p*H to an acid range.[22]

PRODUCTION OF BUTANOL AND BUTYRIC ACID

As mentioned in the section on the genus *Clostridium,* there are numerous species of soil-inhabiting, sporeforming anaerobes which ferment carbohydrate substances with the production of butyric acid, butyl alcohol and other materials of enormous value in drugs, paints, synthetic rubber, explosives, plastics, etc. Some species produce isopropyl alcohol and acetone as well. Important among these organisms are *Cl. acetobutylicum* and *Cl. butylicum*. The true identity of th latter organism seems questionable.

In a later development of the 2.3-butylene glycol fermentation Olson and Johnson had very good yields from the action of *Aerobacter aerogenes* on glucose and on acid-hydrolyzed wheat mashes.[22a]

As in the production of acetone and ethyl alcohol, carbohydrate mashes may be prepared from sugars, starches or cellulosic materials by appropriate processes. In one process, corn is used, after being degermed, ground to meal and autoclaved with water at 30 pounds pressure for at least two hours. This hydrolyzes the starch and sterilizes the mash. After diluting to about 10 per cent corn meal and cooling to around 40° C., the *p*H is adjusted to about 6.0, and a pure culture of the selected organism is introduced with aseptic precautions.

Complete sterilization of all apparatus is essential because many undesirable bacteria of the environment can grow well under the conditions of the fermentation. Particularly troublesome are species of *Lactobacillus*. An organism called *B. volutans*, a gram-positive, nonsporeforming rod, is also especially dangerous.

Fermentation proceeds anaerobically for about three days, when the high wine is removed and subjected to fractional distillation to separate the various products of the fermentation. The nature and proportions of these depend on the strain of organism used, the composition of the mash, the *p*H, and other factors. Normally butyl alcohol, acetone and ethyl alcohol, with carbon dioxide and hydrogen in large amounts, predominate when *Cl. acetobutylicum* acts in a glucose mash. Other substances may occur in smaller amounts. Riboflavin (a vitamin of the B complex), is a valuable constituent of the residue after distillation of the solvents. Butyl and isopropyl alcohol are important among the fermentation products of *Cl. butylicum*, along with butyric and acetic acids.

Relation of Sporulation. The most vigorous and productive fermentations are brought about by cultures of the organisms which hav undergone a series of heat treatments to select vigorous and rapid sporeforming cells. A long process of alternate heatings to kill vege tive cells, and periods of vegetation and sporulation which are chec abruptly by heating again, select the rapid and resistant sporulat survivors. Temperatures of around 100° C. for about one minute used. Thus only vegetative cells and less resistant spores are ki and the rapid producers of very resistant spores are selected for use

PRODUCTION OF LACTIC ACID

Lactic acid is used in a large number of commercial processes, s as the preparation of drugs, acidification of wines for vinegar

duction, pickles, preparation of foods, plastics, lacquers, etc. It is produced by many fermentative organisms from various carbohydrate substrates such as potato or corn starch, molasses or milk whey. The most commonly used organisms are *Streptococcus lactis* and lactobacilli (*L. delbrückii, L. leichmannii, L. bulgaricus*). As in other industrial fermentations, any nutrient deficiencies in the mash must be rectified by appropriate additions of sources of phosphorus, nitrogen, or whatever may be needed. For lactobacilli, the vitamins, pantothenic acid and riboflavin as well as nicotinic acid are requisite additions unless the mash contains these in adequate amount (see section on assay of vitamins with lactobacilli, page 699).

In the United States corn, molasses and whey are commercially practical sources of carbohydrates, as in other important industrial fermentations. Indeed, the general principles illustrated by the processes for producing ethyl and butyl alcohols hold true in lactic acid production, modifications being made in the composition of the mash, time, temperature and *p*H of the process in order to favor the organisms used and to promote lactic acid formation. For the lactobacilli temperatures around 45° C. are used, for *Str. lactis,* about 25° to 30° C. Anaerobic conditions are suitable for both genera. A *p*H of around 5.5 favors growth of lactobacilli; around 7.0 is suitable for *Str. lactis*. Fermentation times vary with substrates, organisms and other factors. Metal vats are not used because of the acidity of the "beer."

Lactic acid production differs from previously described fermentative processes in that the acid is neutralized with calcium hydroxide as fast as it is formed. Otherwise growth would soon cease. The final product in the fermentation vats is therefore calcium lactate which, if desired as pure acid, must be changed to lactic acid by the addition of sulfuric acid which precipitates the calcium as calcium sulfate. Calcium lactate as such has many commercial and medicinal uses.

As in the production of butyl alcohol, etc., precautions are necessary to prevent contamination of the mashes used for lactic acid production. As the anaerobic conditions and substrates favor growth of clostridia, special precautions are taken to avoid these organisms.

These examples serve to illustrate the general nature of pure-culture commercial processes. Regardless of end-products, the same general principles apply to all. The microbiological applications lie in discovering organisms which produce substances of value, the optimum conditions for their growth, and in maintaining pure cultures of maximum productive power. It is obvious that, as Pasteur discovered in the production of wines and beer, there are "diseases" of the various industrial fermentative processes which are due to overgrowth by contaminating organisms. These may suppress the growth of the desired bacteria or produce other acids or alcohols than those desired, or metabolize those sought as fast as they are formed, thus reducing the yield. Special preliminary treatments such as heating or special pure-culture technics may be employed in some processes.

ENZYMES OF MICROORGANISMS IN INDUSTRY

During the recent war there was a greatly increased demand for alcohol to be used for synthesis of rubber and many other purposes. Industrial alcohol production necessitated the use of grain as a source of starch. This in turn created a shortage of malt, the diastatic or amylolytic* enzyme ordinarily obtained from sprouted grain. The malt is used to "convert" or hydrolyze the starch in grain kernels so that it may be fermented by yeast and so changed into alcohol. The shortage of grain malt directed attention to other sources of amylolytic enzymes (amylases). A long-known but until recently little used source of amylases, as well as other enzymes such as proteinases, is the group of molds. Several processes for producing mold enzymes have been developed. Discussion of one of these will illustrate the problems involved.

The Modified "Amylo" Process. The classical "Amylo" process originated in France in 1896 and depends on growing the proper mold in the mash to be fermented, in the final fermentation tank, with vigorous aeration since the molds are strict aerobes. The method required that the whole apparatus, with the huge volume of mash, be sterilized and that pure-culture technique maintained throughout—obviously a costly and troublesome procedure. Erb and Hildebrand[23] modified the procedure by starting seed cultures of the mold (a species of *Rhizopus*) in small flasks in pure culture; using this to inoculate heavily 75-gallon pure cultures, and finally a 2500-gallon aerated tank of sterile medium. This large pure culture serves as the inoculum for the 20,000-gallon fermenter and overwhelms any contaminating organisms.

In the modified "Amylo" process of Erb and Hildebrand granular flour mash is made with 190 pounds of flour, and 2 per cent of the scarce grain malt (the usual proportion is 20 per cent; a saving of 90 per cent of the usual amount of malt per 100 gallons). This mash is not sterilized. The malt acts to a small extent on the starch during a "conversion" period of one hour at 62° C. The mash is thus pasteurized and is then pumped to the fermentation tanks where it receives an inoculum of 4 per cent of pure culture of yeast and the 2,500-gallon pure culture of mold. This mixture is aerated for three to four hours to allow heavy growth of mold to develop and the amylases of the mold to hydrolyze the flour starches. The fermenter is then filled to capacity with the mash and the yeast ferments the hydrolyzed starch. For cultivation of the pure 75- and 2,500-gallon cultures of mold still-waste is used after detoxification with aluminum powder.

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* Amylum = Latin for starch.

In this process the only pure cultures are the yeast and mold inocula. The mash itself is double pasteurized and only a few bacteria survive and these are overgrown in the fermenter and also held in check by certain added bacteriostatic agents.

The process uses, in part, the same principle as is used in the Coblenz method for the Voges-Proskauer test (see page 584), *i.e.*, the development of a very large, ready-formed mass of young, active cells and enzymes, and their overwhelming and rapid action in contact with the substrate. Fermentation is complete in 40 to 55 hours. Were one to inoculate the 20,000 gallons of mash with one loopful of yeast and mold the process might take months and bacterial contaminants would certainly ruin it.

Strains of *Rhizopus* and *Aspergillus* species having special properties with regard to their amylolytic enzymes systems are available from Northern Regional Research Laboratories of the U. S. Department of Agriculture. For example, one strain of *Rhizopus* possesses intermediate maltase activity with negligible alpha-amylase. One strain of *Aspergillus niger* has a high maltase activity and less alpha-amylase, while another has reverse properties. Special studies of the industrial application of some of these strains have shown them to be exceedingly valuable.[24]

Bacterial Enzymes. Both amylolytic and proteolytic enzymes are produced in sufficient quantity by certain bacteria to be of commercial value. A good example of commercial enzyme production is seen in the production of amylase and protease by *Bacillus mesentericus*. The amylolytic enzyme is used in the brewing and related industries to convert raw starches into materials fermentable by yeasts. Amylases are also prepared from *B. macerans* and *B. polymyxa*.[25] In the textiles industry starch partly digested by bacterial amylase is used for sizing cotton fibers, while similar enzymes are used for desizing cotton prior to dyeing and bleaching processes. Sizing pastes for paper are prepared from starch treated with bacterial enzymes. Bacterial protease, present in preparations of *B. mesentericus,* which is both proteolytic and amylolytic (see section on genus *Bacillus,* page 507), also is used in desizing rayon which is treated with gelatin or casein, as well as for degumming raw silk. There are many other uses in industry for bacterial enzymes.

Production. In one process amylolytic and proteolytic enzymes from *B. mesentericus* are prepared in culture medium made from soybean and peanut cake, dextrins, and suitable mineral supplement. The medium is sterilized by autoclaving and inoculated with a selected strain of the organism to be used. Some varieties produce more protease and less amylase, and vice versa. Culture media and conditions also have a determining effect on the relative proportions of the two types of enzyme.

The inoculated medium is run into numbers of shallow trays stacked in a cabinet. This favors aerobic growth in a thin surface film. The amount of aeration and temperature are carefully controlled. From 500 to 1000 gallons of "mash" may be incubated at one time. After about a week at around 30° C. the mash is centrifuged at high speed, and the clarified fluid collected and stored at low temperature or preserved with disinfectants. It may be concentrated by distillation in vacuo. The product is of value in commercial processes operating at a *p*H range between 6.5 and 8, and at all temperatures from around 20° to almost 100° C. In the presence of starch, at *p*H of 7 to 8, it even withstands boiling for a few minutes.

B. subtilis is also used as a source of amylase. It may be cultivated on "still waste" adjusted to a *p*H of 7.0 and inoculated with pure culture. The formation of a pellicle and its integrity are essential to good yields of amylase. It is possible, since the organism is a strict aerobe, to utilize a process similar to the generator process in vinegar manufacture.[26, 27]

Submerged Cultures. In all processes in which wide-surface, shallow culture (small volume) methods are used to secure good aeration, the tendency now is to develop means for tank cultivation with resultant increase in volume of product. Submerged growth during tank cultivation of strictly aerobic organisms necessitates thorough aeration of the contents of the tank by agitation or bubbles of air. This helps remove NH_3 and CO_2 from the culture medium, as well as providing oxygen. Such tank or submerged growth processes are highly developed in the penicillin and streptomycin industries. Very careful adjustments of O-R potentials, amount of mechanical agitation, ratio of dissolved oxygen to other ingredients in the medium, *p*H, etc., are necessary in each process.[28, 29] This is one of the fascinating fields for research in industrial microbiology.

Proteinases. Proteolytic enzymes are used in the brewing industries, as described above, in the leather industry to remove flesh and putrefiable material from hides, to remove protein from rubber latex, silk, cleaning fabrics, etc. A method has recently been described for the production of proteolytic enzymes from the juice expressed from waste asparagus butts. This transformation of garbage into valuable enzymes is riches indeed.[30]

The usual processes for bacterial proteinase production are much like those for amylase production and mostly involve the same organisms. In the asparagus juice process mentioned above similar techniques are used: shallow layers of the prepared juice are used as the medium. The main advantage in such a medium is economic. It is a good illustration of the enormous possibilities for profitable use of wastes. One step in the manufacture of linen involves the action of microorganisms on pectin, the gum which cements together the fibers of the flax plant. The destruction of pectin depends on the enzyme pectinase produced by the organisms. Details of the process will be given later.

Pectinase. Pectinase, the enzyme responsible for *retting* or the hydrolysis of pectin, is formed by a number of bacteria, some of which are used in pure culture under carefully controlled tank procedures and added to the tanks in the form of "starters." Among these are aerobic organisms like *Bacillus subtilis* and a related species called *B. comesii. Clostridium felsineum, Cl. butyricum* and its congeners are used for anaerobic retting. Some of the plant pathogens, as *Erwinia carotovora,* are also active producers of *pectinase,* but are not ordinarily used in retting. If the enzyme could be prepared cheaply in a pure and concentrated form, it might be of great assistance in retting. The same is true of other industrial processes involving bacterial enzymes. Methods of producing pectinase have been devised but are expensive.

SOME FERMENTED FOODS

Turning now to fermentative processes in which pure cultures are not necessary, or in which they are, in practice, seldom used, we encounter a number of procedures which involve microorganisms but usually no special knowledge of bacteriology. Very often, however, the skilled microbiologist is in great demand when trouble occurs in such processes. Vinegar manufacture is one of these processes and has been described.

Ensilage. One of the commonest fermented food products is ensilage. Fermentation of the carbohydrates in green plant tissues, especially by members of the genera *Lactobacillus, Escherichia,* and *Aerobacter* is used constantly (and usually unconsciously) by farmers in the preparation of silage, a food for cattle. Finely chopped, partly mature plants like corn stalks or alfalfa are tightly packed in tall, cylindrical tanks (silos). As fermentation proceeds, the material becomes warm and acid. The heat can be reduced if the rate of oxidation is decreased (exclusion of free oxygen) by tight packing. Oxygen is used up rapidly so that molds and strict aerobes cannot grow, only facultative and strict anaerobes surviving. The acidity prevents the growth of putrefactive organisms. In the first stages probably the *Escherichia* and *Aerobacter* predominate; however, this is undesirable as they produce gas. As acidity increases, they subside and the aciduric organisms (chiefly lactobacilli and streptococci) predominate. These produce lactic acid, with small amounts of butyric, propionic and acetic acids, esters, etc., which give an aroma and flavor to ensilage which is relished by cattle.

After three or four weeks, the process slows and the fermented mass gradually cools. Carbon dioxide is produced during the process and often settles in the lower part of silos so that a person ignorant of this may die if he stays in the depths of a poorly ventilated silo.

It has been claimed that fermentation in silos may be facilitated by introducing cultures of various fermentative bacteria such as *Streptococcus lactis* or *Lactobacillus sp.,* as the material is packed. However, this is seldom done in practice. Various other organisms are doubtless involved, including the bacteria (micrococci, sporeformers, *Pseudomonas,* etc.), of the soil. In some sections of the country molasses is added to promote fermentation by the acid formers, and improve palatability but this is not a common practice.

If too much soil is introduced with the fodder undesirable and excessive putrefactive processes spoil the product. For example, butyric acid organisms like *Clostridium butyricum* get in and ruin the silage. The action of such organisms would constitute a "disease" of silage. *Cl. botulinum,* a soil anaerobe, forming a deadly poison, has also caused much damage to livestock by its growth in silos.

Sauerkraut. The production of this savory delicacy is dependent upon the acid fermentation of cabbage by the bacteria normally present on the plants. These include the lactobacilli, soil streptococci and other soil bacteria. The desired fermentation is principally lactic.[31] Pure cultures of lactobacilli are sometimes used to aid the process.[32] Commonly, however, the fermentation is allowed to proceed naturally. Salt is placed between the layers of shredded cabbage as it is packed and this prevents the growth of many undesired bacteria and also draws out the juices of the cabbage. Except for the salt, sauerkraut is quite analogous to silage. Sometimes "sauerkraut" is prepared by merely cooking cabbage in vinegar. Such a product lacks the true, rich flavor of fermented sauerkraut because the natural fermentation produces many organic esters, etc., besides acetic and lactic acids, which add greatly to the aroma and flavor.

Making Sauerkraut at Home. Directions for domestic sauerkraut will illustrate methods of applying principles used in commercial practice.[33] Large, solid mature heads of cabbage are allowed to wilt for one or two days, so that long shreds may be cut. Any soil or foreign matter must be removed. The shredded cabbage is packed in clean 5-gallon jars or barrels which have previously been paraffined on the inside. Contact with wood may darken the kraut. Salt is sprinkled lightly in, as the packing proceeds, in the proportion of about 2 to 2.5 per cent of the total weight. The final concentration of salt is around 2 per cent.

This determines the bacterial flora to a great extent. Too much salt may darken the kraut or interfere with fermentation. Too little will permit spoilage by soil bacteria. The kraut is firmly pressed down with a wooden tamper, care being taken not to bruise it, the object being merely to force out the air. Air pockets permit spoilage bacteria to grow and darken the product. Bacteria begin to ferment the carbohydrates in it. When the container is full, the juice should just cover the cabbage. The kraut is covered with a clean muslin cloth and then a paraffined cover just small enough to fit inside the container is put on. A weight may be placed on the cover so as to keep the kraut just below the juice, especially when gas bubbles begin to form. After two to four weeks at summer temperatures, fermentation is complete and the kraut is ready to ship or consume.

If not used at once, it is best heated to about 125° F., put up in jars or cans and finally heated in boiling water for about twenty minutes, after which the containers are sealed and placed in a cold room.

Bacteriology of Sauerkraut. Since the microbial flora normally present on the cabbage leaves is allowed to grow as it can, the bacteriology of sauerkraut fermentation is complex. The conditions of packing provide a rather selective medium so that only certain species are very active. Those sensitive to more than 2 to 2.5 per cent salt content are inhibited, as well as the strict aerobes. As in silage a few members of the *Escherichia* and *Aerobacter* groups may start to grow. These are highly undesirable, however, and every effort should be made to avoid admitting them to the kraut in excessive numbers. Ordinarily, unless spoiled or dirty cabbage is used, acid formation begins almost immediately and increases rapidly, at temperatures around 70° F., and only facultative, anaerobic, aciduric and acidophilic forms can survive. During the first two to five days, certain species of streptococcus-like organisms, somewhat like *Str. lactis,* are found in great numbers. These belong to the genus *Leuconostoc, L. mesenteroides* being common. Later, these die off, because of the acidity, and organisms of the genus *Lactobacillus,* because of their greater resistance to acid, gain the ascendancy. Species such as *L. plantarum* and *L. brevis* (pentoaceticus) are commonest, in the order named. The latter is most aciduric and predominates finally. Acetic and lactic acids, carbon dioxide and alcohol are produced in considerable amounts. Other acids and esters give pleasant aromas and flavors. Cool summer temperatures around 70° F. favor the best fermentations. Higher temperatures may induce darkening due to abnormal fermentation.

Pickles. Fermentation by mixtures of organisms normally present on certain vegetables, in a manner analogous to the manufacture of sauerkraut, is an important part of the production of pickles, ripe olives and the like. In making pickles commercially, selected and sorted cucumbers are placed in large wooden vats and covered with brine solution of from 8 to 10 per cent concentration. A framework of wood is placed over the pickles to prevent their floating. As water is drawn from the vegetables by the brine more salt is added gradually over a period of about 6 weeks till the concentration is increased to 16 to 18 per cent.

During the summer, *i.e.,* when cucumbers are ripe, temperatures range around 28° C. Under these conditions, and at the salinities provided, active growth of *Lactobacillus plantarum* occurs. Most other organisms are inhibited. This results in the production of lactic acid which, with the action of the brine, changes the color, consistency and flavor of the cucumbers. After about six weeks the vat may be emptied and the pickles packed in fluid containing sugar and various flavorings, spices, etc.

It is evident that halophilic organisms capable of growing at temperatures of 25° to 30° C. will predominate. If temperatures are low, around 20° C., and the salinity only about 5 per cent undesired slimy growths may occur. Organisms such as *Leuconostoc* and sporeforming bacilli and others may then ruin the pickles. This is in contrast with the fermentation of sauerkraut, in which the salinity is around 2.5 and the temperatures around 20° C. The predominating organisms are *Leuconostoc* and *Lactobacillus plantarum* and *Lact. brevis.*

The bacterial flora of fermented vegetable products of these types appears to depend more on salinity and temperature than upon the vegetable product involved.[34] Methods for the bacteriological examination of pickled vegetable products have been described by Etchells and Jones.[35]

THE VEGETABLE FIBER INDUSTRY

Flax, Hemp, etc. The leaves or bark of flax and hemp plants are prepared for use by allowing bacteria which attack *pectin* to act upon them. Pectin is a plant gum which acts as a sort of glue or cement, holding plant cells, tissues and fibers together. When it is hydrolyzed by bacteria, as in the preparation of jute, hemp or flax for manufacture, the bast fibers of the plants are loosened and freed from the nonfibrous parts and are then ready for further treatment. The process is called "retting" and is of two kinds, "dew retting" and "steeping" or "water retting."

In *dew retting* the fibrous plant material is spread about in piles in meadows. The dew keeps it moist and the natural bacterial action proceeds. The piles of plant stalks or leaves are usually left exposed to the weather all winter, or from 2 to 5 days in warm weather. Unduly warm weather may cause excessive bacterial action, especially by cellulose-digesting molds and bacteria, like *Aspergillus, Cellvibrio, Cellfalcicula* and various species of *Clostridium,* which may weaken or entirely ruin the fiber.

Steeping or water retting may be carried out in slow flowing, warm streams (Fig. 327). The shallows of the Nile have served for retting flax for centuries. The rate and effectiveness of the process depend on the nutrient substances in the water, its temperature and the bacterial flora. As in dew retting, there is little control over the process.

Tanks or vats are preferable and are used on a large scale in some areas. An aerobic or anaerobic flora may be made use of since pectin-digesting species of either group exist. In the anaerobic vat process the vegetable material is placed in the vat which is then filled with water. Soluble nitrogenous and carbohydrate extractives from the plants pass into the water, along with mineral and other nutrient compounds, and constitute a suitable solution for the growth of bacteria. The normal flora of the plants grow in the solution, first the aerobes, which use up the oxygen, then the anaerobes. These include such organisms as *Clostridium butyricum* and related soil species, important among which, in this connection, is *Cl. felsineum* which is particularly active in pectin digestion. The process goes on well at warm summer temperatures (around 30° C.). The time is important, as over-retting may permit excessive action of cellulose digesters which ruin the fibers. About two to three days at 30° C. produces satisfactory results.

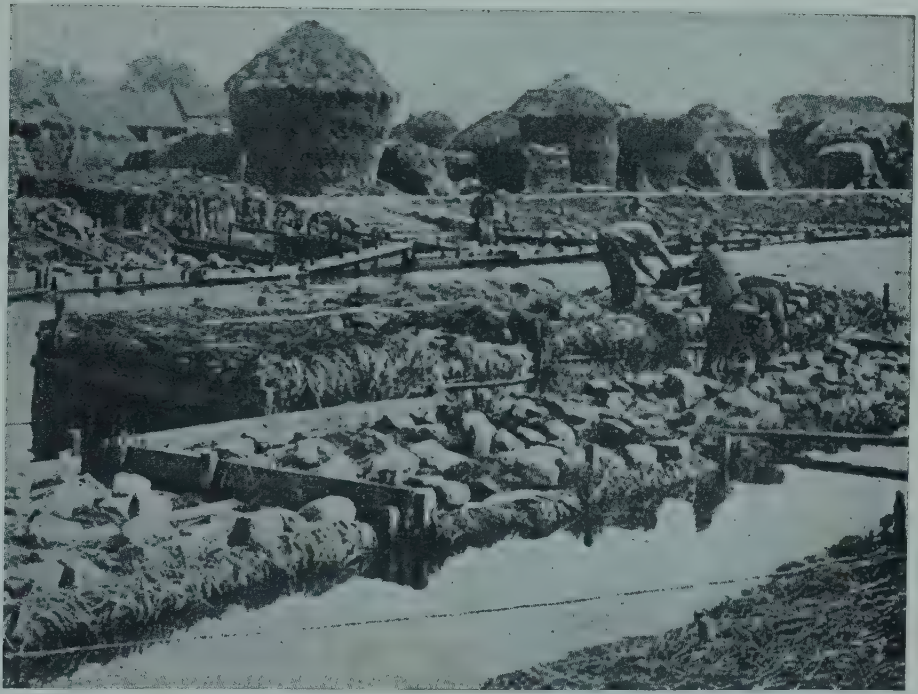

Fig. 327. Flax retting in a stream. (W. D. Darby, "Linen, The Emblem of Elegance," Dry Goods Economist, Copyright by Textile Publishing Co.)

In the aerobic process, air is bubbled through the tank and aerobic pectin digestion takes place. *Bacillus macerans* is important in this process. It is said to yield a superior grade of flax fiber.

MICROBIOLOGICAL ASSAYS

It has already been explained (Chapters 8, 14) that, while primitive organisms may synthesize amino acids, vitamins and other substances essential to their growth, more highly evolved mutants often are absolutely deficient in respect to the synthesis of such substances. In a medium complete in all nutritional respects except for the absence of one of these essential growth factors (sometimes called nutrilites), growth will not occur. If a minute amount (say 0.01 gamma per ml.) of the missing substance be added, in some food being tested for vitamins, some growth will occur. More growth will occur in the presence of more of the missing factor. Up to a certain point growth bears a definite ratio to the amount of that specific growth factor in the "unknown" food added to the medium. By accurately measuring this growth, and comparing it with growth obtained in solutions containing the same growth factor *in known concentration* it is possible to estimate closely the concentration of the growth factor in the "unknown" material. This method of estimation of growth factors is spoken of as *microbiological assay*.

Certain organisms lend themselves very well to such assay procedures. *Lactobacillus casei* and *L. arabinosus* are easy to cultivate, relatively hardy, harmless, and deficient in a number of growth factor syntheses, including at least 16 amino acids (valine, leucine, glutamic acid, etc.), riboflavin, biotin, pantothenic acid, nicotinic acid, etc. Other organisms may be used for assay of other substances: for example, yeast for thiamine; *Streptococcus lactis* for folic acid; *Neurospora sitophila* (a pyridoxine-deficient artificial mutant) for pyridoxine; *Lactobacillus delbrückei* for aspartic acid, etc.

The production of growth-factor deficient mutants by ultraviolet irradiation offers almost limitless possibilities for this sort of work (Chapter 8).

Assay Methods. The basic principle of all of the assays is the same. Four different methods of measuring growth of the test organisms may be cited.

1. *Measurement of gas* (*CO_2*) produced during fermentation of sugar in the test culture by yeast. This is routinely used in the microbiological assay of thiamine.
2. *Measurement of acid production by Lactobacillus*, or other bacteria, from glucose in the test medium. This is commonly used in assays of nicotinic acid, riboflavin, etc.
3. *Measurement of turbidity* due to bacterial growth, using a photo-

electric turbidimeter. This may be used with *Lactobacillus,* or with *S. lactic* in folic acid assay, or other assays.

4. *Gravimetric measurement of the mycelia of molds* like *Neurospora* after removal from the test culture and drying. This is used in pyridoxine assays.

Complete details of procedures can be obtained from the literature on the subject.[36–39] A few examples may be given here to illustrate the general methods.

Pyridoxine Assay with Neurospora. One gram of the material to be tested is placed in a 125-ml. flask with 50 ml. of water. In the case of some substances, as rice bran, 1/N HCl is used in place of water. The material is autoclaved. Thiamine, which interferes in the assay, is destroyed by treatment with sodium sulfite at a *p*H of 8 and temperature of 100° C. The basal medium consists of

Water	1,000.0	ml.
Sucrose	30.0	gm.
Ammonium tartrate	10.0	gm.
KH_2PO_4	5.0	gm.
$MgSO_4.7H_2O$ (20 mg. per ml.)	50.0	ml.
NaCL (10 mg. per ml.)	20.0	ml.
$CaCl_2$ (10 mg. per ml.)	20.0	ml.
$FeCl_36H_2O$ (1.666 mg. per ml.)	10.0	ml.
$ZnSO_4.7H_2O$ (1 mg. per ml.)	2.0	ml.
Biotin (1 microgram per ml.)	8.0	ml.

The medium is distributed in 50-ml. flasks in 5-ml. amounts. Graded amounts of standard pyridoxine solution are added to series of 10 flasks in duplicate, and graded amounts of the "unknown" solution are added to another series of 10 flasks in duplicate. The volumes in all flasks are brought to 10 ml. with water; the flasks are plugged, and then autoclaved. When cool, each is inoculated with 1 drop of a suspension of spores of *N. sitophila* ("pyridoxineless" mutant). Incubation is continued at 30° C. for five days.

The flasks are then steamed to kill the mold. The entire growth is removed, dried and weighed. The weights correspond closely with quantities of pyridoxine if the flasks do not contain excessive amounts of the growth factor.

Assay of Vitamin B_1 by Yeast Fermentation. The rate of fermentation of glucose to carbon dioxide and alcohol by the yeast *Sac. cerevisiae* is largely influenced by the amount of thiamine (B_1) available. In a synthetic medium this vitamin may be supplied or withheld at will and the effect of exact amounts of thiamine determined by measuring the CO_2. By the addition to the synthetic medium of graded amounts of various "unknowns" such as foods, these may be tested for thiamine content. Certain related compounds also found in various foods have an action similar to thiamine. These must be measured separately in a sample of the unknown treated with sodium sulfite which destroys the thiamine but not the interfering substances.

Similar problems are involved in other assays, such as that of biotin.[40] The difference between results obtained in fermentation tests with the treated sample and the untreated sample represents true thiamine. The CO_2 volumes are then compared with parallel experiments in which known amounts of pure thiamine were used, and the thiamine content of the food is easily computed.

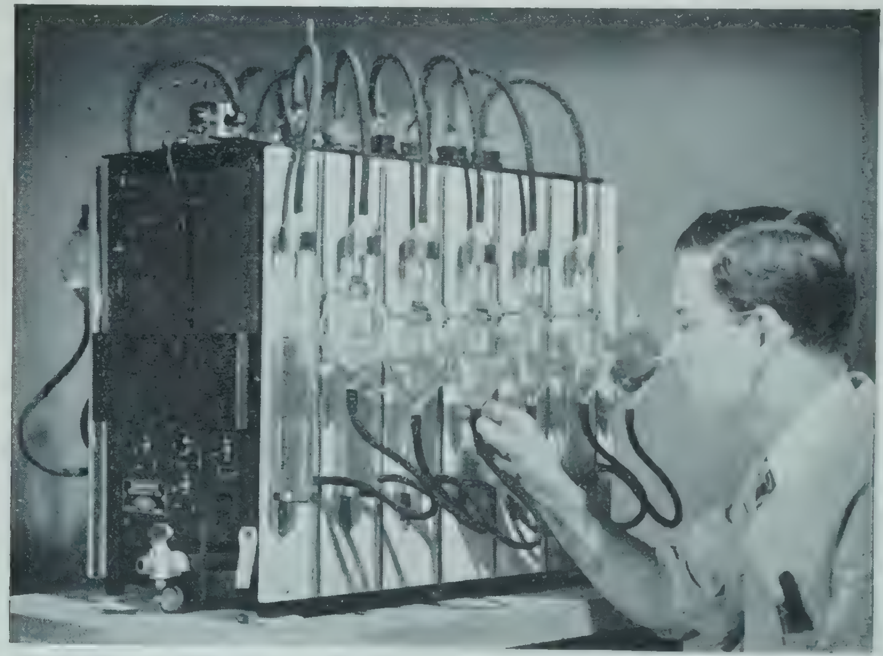

Fig. 328. Yeast machine for thiamine or vitamin assay by method of Schultz. (The Fleischmann Laboratories, Standard Brands Inc.)

In preparing materials for the assay, as described by Schultz and his co-workers,[36] one prepares a buffer solution:

(A)	
Water	1000.0 ml.
$NH_4H_2PO_4$	180.0 gm.
$(NH_4)_2HPO_4$	72.0 gm.
Nicotinic acid	0.2 gm.

Sterilize at 100° C. for 30 minutes.

and the medium (B)

Glucose	200.0 gm.
$MgSO_4$	0.1 gm.
KH_2PO_4	2.2 gm.
KCl	1.7 gm.
$FeCl_3$	0.01 gm.
$CaCl_2$	0.5 gm.
$MnSO_4$	0.01 gm.

The yeast inoculum consists of 10 gm. of bakers' yeast (not the edible cakes) freshly suspended evenly in 200 ml. of water.

Of the medium (B) 7.55 ml. and of buffer (A) 2.5 ml. are placed in 125-ml. flasks held in a water bath at 30° C. Arrangements are made for shaking and for collection and measurement of the evolved carbon dioxide. Machines are available for this purpose (see Fig. 328). The sulfite-treated unknown is placed in one flask, the untreated "unknown" in another. The volume of each is made up to 40 ml. with distilled water, 10 ml. of the yeast suspension are added, and the flasks are closed *quickly*. Incubation and shaking continue for three hours, and gas-volume readings are made in gas burettes attached to the fermentation flasks. From readings of the two unknowns, compared with readings of similar flasks containing known graded amounts of thiamine, the amount of thiamine in the food is computed.

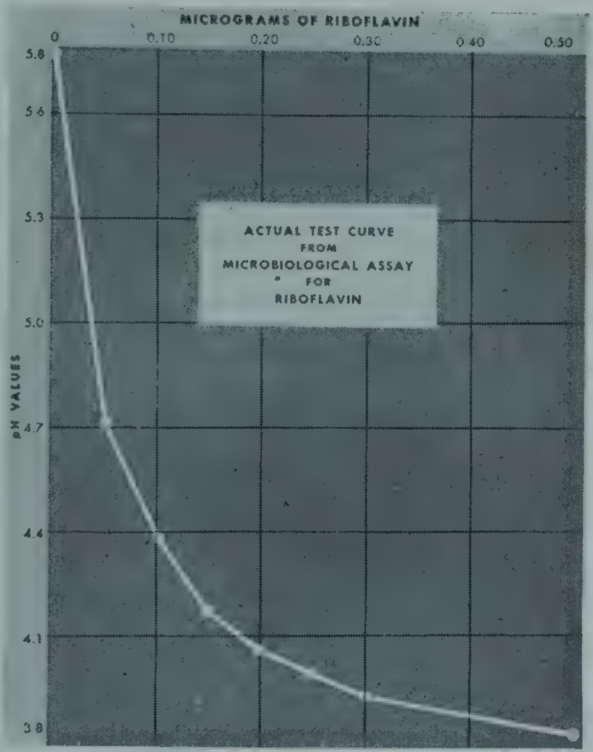

Fig. 329. Microbiological assay for riboflavin. (Courtesy Eli Lilly.)

Pantothenic Acid. A basal medium for pantothenic acid assay contains hydroxide-treated peptone, sodium acetate, cystine, asparagine, riboflavin, 1 per cent glucose, and yeast supplement, as well as suitable minerals including phosphates, iron chloride, magnesium sulfate, etc. It is deficient in pantothenic acid.

The medium is tubed in 5-ml. amounts. The foodstuff to be tested is prepared by fine chopping, mincing and extraction and other means appropriate to the material involved and added in graded amounts to 10 of these culture tubes in duplicate rows. Tubes containing graded amounts of pantothenic acid solution of known strength in place of the food serve for standards of comparison. The total volumes are made up to 10 ml. All are sterilized, and a drop of heavy culture of *Lactobacillus* washed free of culture medium and suspended in saline solution is added to each. After about seventy-two hours of incubation, the acid in all tubes is titrated, plotted on charts, and the pantothenic acid content of the "unknown" determined by comparison with the standards. The method is accurate and saves much time and money as compared with the use of rats and chicks (Fig. 329).

Turbidimetric methods may also be used in place of acidimetry, and are specially applicable in folic acid determinations using *S. lactis*. Except for composition of the medium and slight differences in the quantities used, the procedures are analogous.

In the assay of nicotinic acid with *Lactobacillus arabinosus* casein hydrolysates are used as source of nutrient amino acids. Other sources, as charcoal-treated peptone, acid-hydrolyzed or pancreatin-digested caseins will serve very well.[41] Cystine must be added to all of them. Various other improvements in methods and materials are constantly being devised.[42, 43]

Other Processes. Use of "By-products." A considerable number of other processes of industrial importance depend on the action of microorganisms, but a detailed treatment of them here would be impossible. Some of them are not thoroughly understood. Oxalic acid, citric acid,[29, 44] glycerin, amyl alcohol, humus, acetone, many substances used in paints, drugs, photography, and a long list of other substances and processes are produced commercially by microorganisms. Waste products such as sawdust, shavings, garbage, beet-pulp and, in fact, almost any fermentable or putrescible substance, can be made to yield valuable products by treating them with the proper bacteria, yeasts or molds. The possibilities are limited only by the extent of man's knowledge of microorganisms and his ingenuity in making use of them. Even combustible gases consisting of methane and hydrogen can be made, but the bacterial processes cannot at present compete with natural or coal gas. The use of waste carbon dioxide from alcohol manufacture is a valuable industry in itself.

INDUSTRIAL SPOILAGE

In contrast with the useful activities of bacteria, a word may be said of their destructive action. Several causes of industrial spoilage ("diseases" of fermentations) have been mentioned in this chapter and in the chapters on soil, food, and water bacteria. *Micrococcus*, *Alkaligenes, Flavobacterium, Serratia,* yeasts, molds, etc., are common causes of spoilage. Each type of product is attacked by certain species of microorganism which can metabolize the substance especially well. For example, spoilage of cellulosic products, such as wood, paper, fibers, tobacco, cotton, etc., is brought about by cellulose decomposers like *Cellulomonas, Cytophaga* and anaerobic organisms of the soil. Fermentable substances like syrups, beverages, etc., are attacked by yeasts, lactobacilli, organisms of the coli-aerogenes group and various environmental bacteria including the genus *Clostridium.* Spoilage of proteins like meats, fish, milk and so on results from the action of putrefactive species such as *Pseudomonas, Bacillus, Proteus, Micrococcus, Clostridium* and many others. ZoBell and Grant have shown that rubber may be attacked by certain bacteria.[45] He and co-workers have also shown the important role of bacteria in the destruction of petroleum.[46]

A good example of an industrial "disease" due to a specific organism, and of a method of studying a specific spoilage problem is seen in the demonstration by Tabachnik and Vaughn of the cause of tartrate destruction by organisms closely related to *Cl. butyricum* in tartrate recovery plants. The organisms were found in the spoiled tartrates, in the machinery and in soils adjacent to the plant.[46a]

Prevention of spoilage depends on maintaining conditions unfavorable to organisms which can grow on or in the particular product involved. This may involve drying, refrigeration, aeration, the use of inhibitory salt, sugar or acid concentrations, radiation with ultraviolet light, exposure to sunlight, treatment with substances like creosote, sodium benzoate and the like. Dimond and Horsfall have suggested a method of preventing bacterial spoilage of rubber.[47]

The action of molds on military supplies caused much trouble in war theaters of the Pacific. For example, besides causing rot of fabrics, ropes, etc., they grow inside electrical equipment, optical instruments and other enclosed spaces. Fungistatic vapors were used to control them.[48]

Each type of spoilage is a problem in itself and research in such fields, as well as in the field of productive microbiology, will prove interesting and may prove lucrative to those sufficiently interested to make a special study of the matter. A thorough knowledge of selective biostatic methods is valuable in this field.

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Chapter 40

BACTERIAL DISEASES OF PLANTS

Many organisms cause diseases of plants: fungi, protozoa, bacteria and viruses. The field of phytopathology is a large one. Viral diseases are discussed briefly farther on in this book. In the present chapter we deal with diseases caused by bacteria similar in many respects to the Enterobacteriaceae and *Pseudomonas*. They are included in the genera *Xanthomonas* (formerly *Phytomonas*) and *Erwinia*.

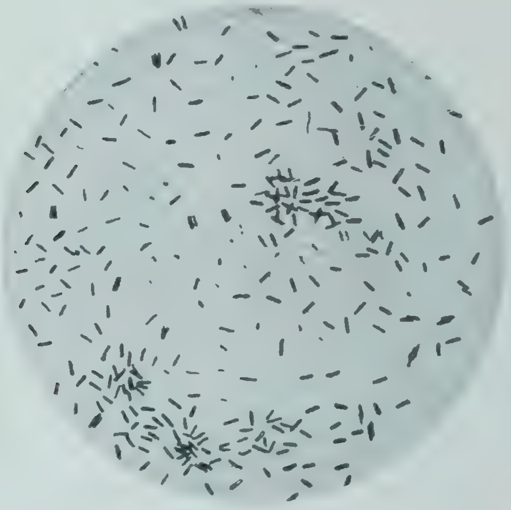

Fig. 330. *Erwinia carotovora* (× 900).

General Characters. Characteristics common to these genera are: (1) a small, straight, rodlike structure, and dimensions usually not exceeding 10 microns in length and 0.5 to 1 micron in diameter (Figs. 330 and 331); (2) none forms spores; (3) all grow well on ordinary laboratory media such as extract or infusion agar or broth, or on rather simple, mainly inorganic solutions in which the only source of energy or nitrogen is an organic compound; (4) all are gram-negative and aerobic or facultative. Some are motile; others, nonmotile.

The genus *Xanthomonas* includes about fifty species which in many respects resemble the *Pseudomonas*. The *Agrobacterium* are very similar to *Xanthomonas*. The line of demarkation between *Pseudomonas* and *Xanthomonas*, where one genus borders on the other, is not always clear. There are plant pathogens in each genus. The pigment of *Xanthomonas* is yellow and not water-soluble, as is the greenish-yellow pigment of *Pseudomonas.* Most of the *Xanthomonas* species are motile, and they form white or yellowish, opaque, butyrous colonies on agar surfaces. They are moderately active and versatile in utilization of various organic compounds; many liquefy gelatin, and otherwise resemble *Pseudomonas.* The type species is *Xanthomonas hyacinthi,* which produces yellow rot of hyacinth bulbs.

The genus *Erwinia,* containing over 20 species, is placed in the family Enterobacteriaceae because the *Erwinia* possess so many properties in common with the latter. *Erwinia* do not inhabit the intestine. The power to digest pectin, as important means of producing diseases of plants and formerly thought to distinguish *Erwinia* from closely similar intestinal genera such as *Aerobacter* and *Escherichia* is common in the last two genera also. Both *Pseudomonas* and *Xanthomonas* are monotrichous while *Erwinia* are peritrichous like the Enterobacteriaceae.

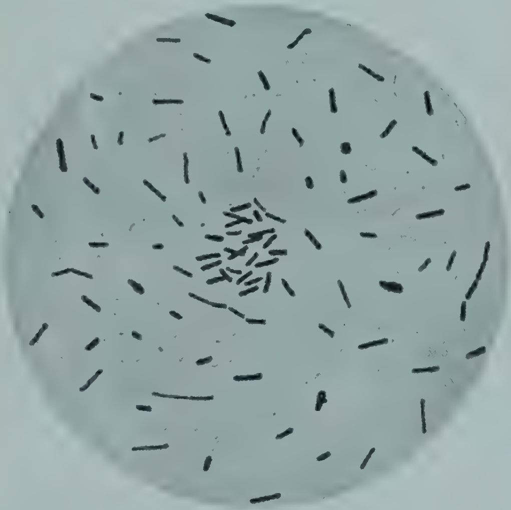

Fig. 331. *Xanthomonas campestris* (× 900).

Further, some species of *Erwinia* have been shown to have antigens found also in many *Shigella* species, suggesting a common origin for *Shigella* and some *Erwinia.*[1] Also, some *Erwinia* species have the power to reduce trimethylamine oxide, an important character of certain groups of *Shigella.*[2] The type species is *Erwinia amylovora,* which produces a soft rot of carrots.

PLANT DISEASES

That bacteria may infect plants, causing diseases which are highly injurious or fatal, was not generally realized until the careful work on this problem done by Erwin F. Smith.[3] The first name of this scientist has been given to the tribe Erwineae and to the genus *Erwinia.*

Diseases in plants present several points of similarity to diseases of animals and man. First, they are specific. A given species of microorganisms usually produces only one disease in a given plant while only certain species of plants are susceptible to a given disease. It is not difficult to imagine why some species of plants, like beans, for example, are not ordinarily subject to infection by the organism causing soft rot of carrots. The chemical and physical conditions favoring growth of the bacteria in the one are absent in the other. In some cases this is due to acidity of the plant sap, or the presence of inhibitory substances such as anthocyanin or tannin. An analogous host-parasite relationship is found in animal diseases. Horses, for example, never have typhoid fever, nor do birds suffer from swine influenza.

The mechanism of resistance in plants, however, is more limited than that in animals. Animals have, in addition to the differences in chemical and physical make-up of species, *leukocytes,* and *antibodies* which circulate in the blood. Plants have neither leukocytes nor blood circulation and must depend on local resistance or species immunity. However, it has been shown that plants, parts of which have been affected by certain diseases, may produce immune outgrowths, and that susceptible species, grafted onto recovered plants, become immune and remain so through many successive propagations, suggesting an active immune process.[4]

In many instances the microorganism causing a plant or animal disease can infect the plant or animal only through some definite natural portal of entry. This is also true of animal diseases. In plants this may be the stomata* of the leaves, the nectar ducts of the flowers, or an abrasion or wound. The intact outer bark and epidermal cells prevent the entrance of many parasites.

Some individuals of many species of plant seem to be more resistant than their fellows to invasion by the microorganisms of a given disease and it has been found possible, by selecting and breeding these individuals, to develop resistant varieties. The same process doubtless occurs in nature and is known to function in animals. It is known as natural selection.

Pathogenesis of Plant Diseases. The local protections of plants are sometimes of little avail against the properties of some microorganisms. For example, the *Erwinia* secrete enzymes (as pectinase) which digest the tissue-connecting substances of the plant (pectins, etc.), causing these to break down, so that the tissue cells, no longer cemented together by the pectin (middle lamellae), fall together into a slimy mass. The bacteria also produce enzymes capable of making available for their metabolism the plant cell walls, protoplasm and juices. Thus they are enabled to invade and multiply within the host plant, parasitizing it and destroying various parts, or so interfering with its growth and physiology that it is greatly injured or killed. All this is entirely analogous to animal and human disease.

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* Microscopic openings in the under surfaces of the leaves through which pass air and water vapor.

Types of Plant Disease. *Rots.* Plant diseases vary in type, depending on the bacterial invader, on how and where the invading organism attacks, and on the anatomy and chemistry of the plant. Those bacteria capable of digesting the pectins in tissues are likely to produce "rots" of various kinds; soft, hard, wet, dry, stem, root, and so on. *Erwinia carotovora* affects carrots and a number of other plants in this way (Fig. 332). Various species of *Erwinia* are most commonly involved as the cause of plant "rots." *E. carotovora* is a very vigorous producer of pectinase.

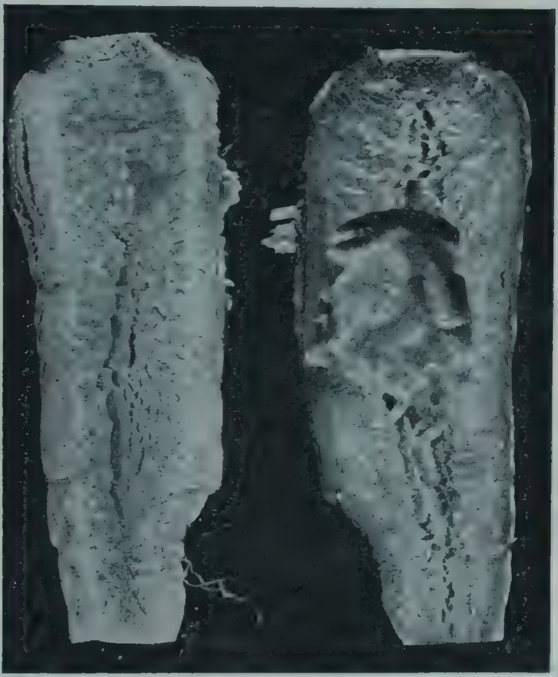

Fig. 332. *Erwinia carotovora,* streaked on raw carrot, which was for three days kept at 23° C. in a large covered dry culture dish. The inoculation was from a rotting raw potato which had been inoculated from gelatin colony. The carrot was first washed, then soaked in 1 : 1000 mercuric chloride water, and cut with a cold sterile knife. The left (uninoculated) part remained sound. (Erwin F. Smith.)

Rots caused by true pathogens which attack and digest healthy *growing* tissues must be distinguished from spots of decay of *dead* tissue due to common saprophytes of the soil, which start to grow in a part of the plant which has first been injured by some other means. These organisms produce ammonia or other poisonous substances which kill the plant tissues nearby, thus permitting further growth and advance of the rot. They do not initiate the rot. They are "opportunists." The action of certain saprophytic organisms as secondary invaders of lesions of animals and man is closely analogous.

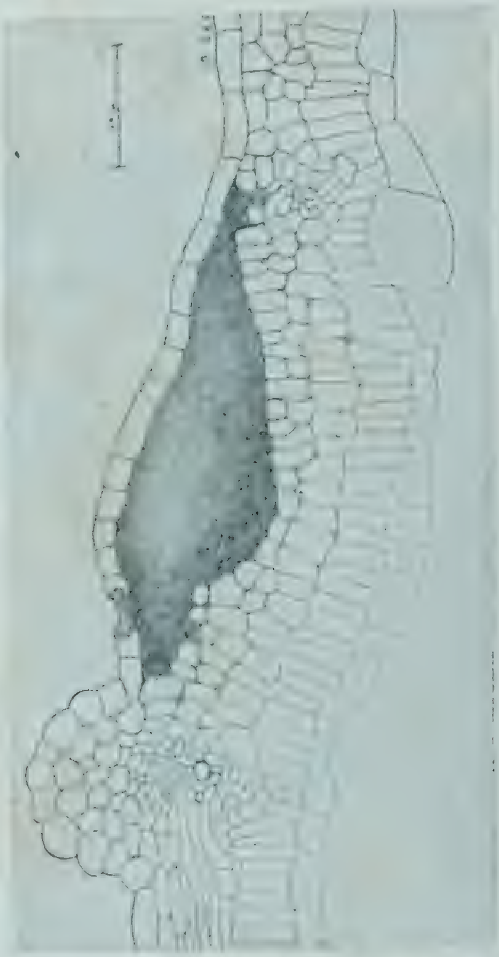

Fig. 333. South African mulberry blight due to *Pseudomonas mori:* section through a water-soaked spot on a leaf of the common mulberry which was fixed five days after inoculation, showing lower epidermis (left side) lifted, a large bacterial cavity (dark area) and penetration of the intercellular spaces of the mesophyll (center area) by the bacteria. (About 100 ×.) (Ethel M. Doidge.)

Blights. Bacteria which invade the spaces between the cells and grow in the plant juices are likely to cause discoloration or death of whole leaves or branches due to the stoppage of sap flow or to poisons (toxins) given off by the bacteria (Fig. 333). Such conditions are found in the "*fire blights*" of pears, apples and other pomace-

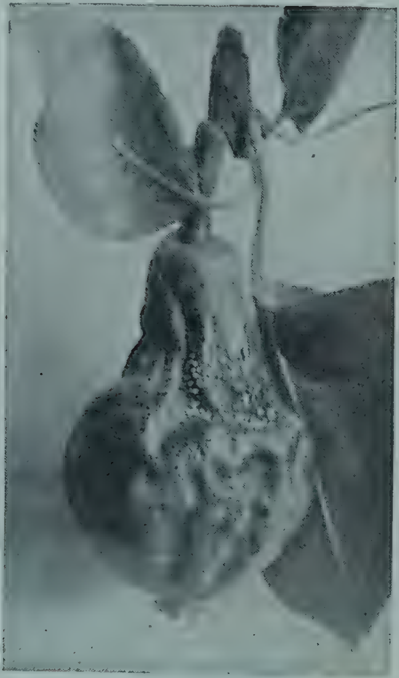

Fig. 334. Pear fruit showing effect of invasion by the fire-blight organism. The pearly beads on the surface are drops of exudate containing large numbers of bacteria. (After Jackson, from Owens, "Principles of Plant Pathology," John Wiley & Sons, Inc., publishers.)

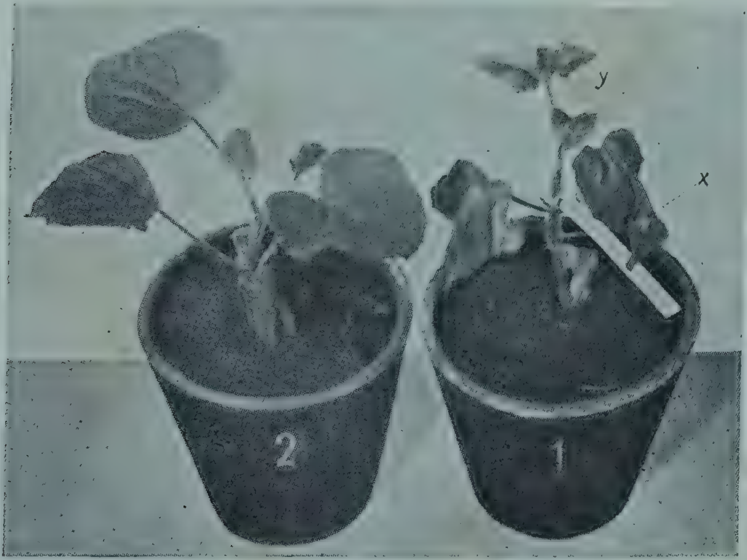

Fig. 335. Young melon plants: No. 1, inoculated at *X* and *Y* with *Erwinia tracheiphila;* No. 2, healthy control. (E. F. Smith.)

ous fruits (Fig. 334). *Erwinia amylovora* is one of the species causing such diseases, although various species of the *Xanthomonas* are also frequently responsible for this type of plant pathology.

Wilts. Another type of disease due to invasion of the tissue spaces is exemplified by the so-called "*wilt*" of cucumbers, melons, squashes, etc., caused by the growth of *Erwinia tracheiphila.* The masses of bacteria become so dense in the sap vessels and spaces that flow of sap is obstructed and the plant wilts (Fig. 335). A wilt of corn is due to *Bacterium* (Xanthomonas?) *stewarti.*[5]

Spots. A number of plants are subject to a type of disease called *leaf spot* or "black spot." In such diseases the bacteria do not invade the tissues extensively, their destructive effect being localized around their portals of entry, which are usually the stomata. The diseased area in some types of leaf spot drops out, leaving a small "shot hole." "Leaf spot" of peaches or cotton is a good example of this type of disease (Fig. 336). The *Xanthomonas* are chiefly involved in producing leaf spots in various plants. Blister spot of apples is caused by *X. syringae.*[6]

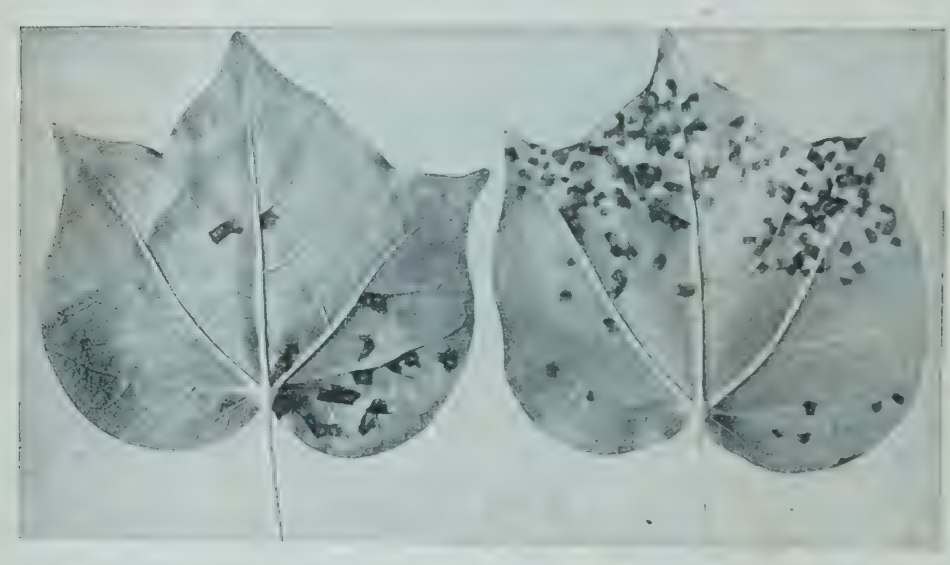

Fig. 336. Inoculated cotton leaves showing angular spots due to stomatal infection by *Xanthomonas malvacearum.* Bacterial suspension sprayed on. Spots in second stage, *i. e.*, beginning to shrivel and brown. Time, six weeks. One half natural size. (Erwin F. Smith.)

Abnormal Growths. A very common warty or cancer-like overgrowth of plant tissues, called *crown gall* or *root gall,* is caused by bacteria (Fig. 337). Galls occurring on a wide variety of plants are due to *Agrobacterium tumefaciens.*[7] The organisms grow in the tissues and irritate the cells so that an abnormal or pathological overgrowth is produced. This is very suggestive of human cancer, but differs from it in many fundamental respects. Erwin F. Smith advanced a number of reasons for believing that plant galls and human cancer are of similar nature, but students of cancer have not agreed with him. The cause of human cancer is not known.

The power to cause hypertrophies (extra growths) in plants is not confined to *A. tumefaciens*. There is, for example, the formation of root nodules by *Rhizobium* species (see page 605), in the symbiotic fixation of atmospheric nitrogen. The genus *Agrobacterium,* since most species cause galls, is placed in the family Rhizobiaceae. *A. tumefaciens,* which causes galls, does not fix nitrogen as do species of *Rhizobium*. Apparently here is another spectrum of organisms intergrading by various overlapping characters, all closely related to the production of hypertrophies in plants. However, within the genus *Xanthomonas,* several well defined groups have been shown to exist, differentiated by their antigenic structures.[7a]

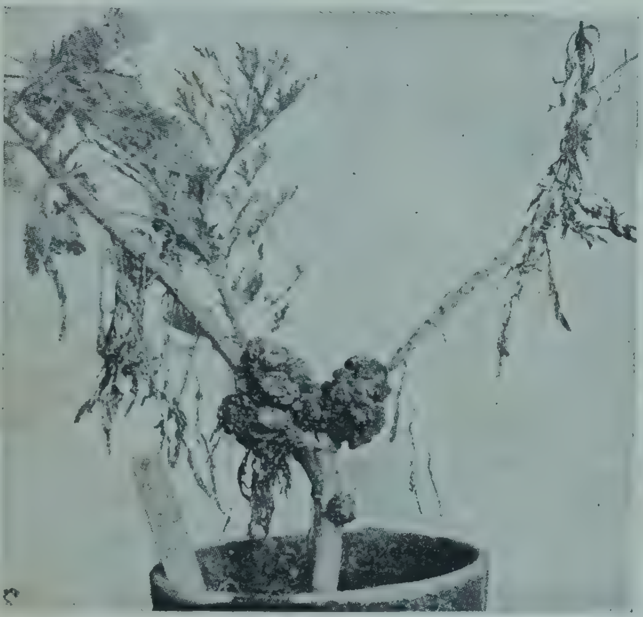

Fig. 337. Crown gall on white Paris daisy (*Chrysanthemum frutescens*). Plant inoculated with a pure culture of *Agrobacterium tumefaciens* and photographed seven months later. One branch killed. About one quarter natural size. (Erwin F. Smith.)

Agrobacterium Radiobacter. A species occupying a rather anomalous position in this assemblage is *Agrobacterium radiobacter*.[8, 9] This so closely resembles the rhizobia and *Agrobacterium tumefaciens* that it is with difficulty distinguished from them.

However it fails to produce nodules in, or to invade the roots of,

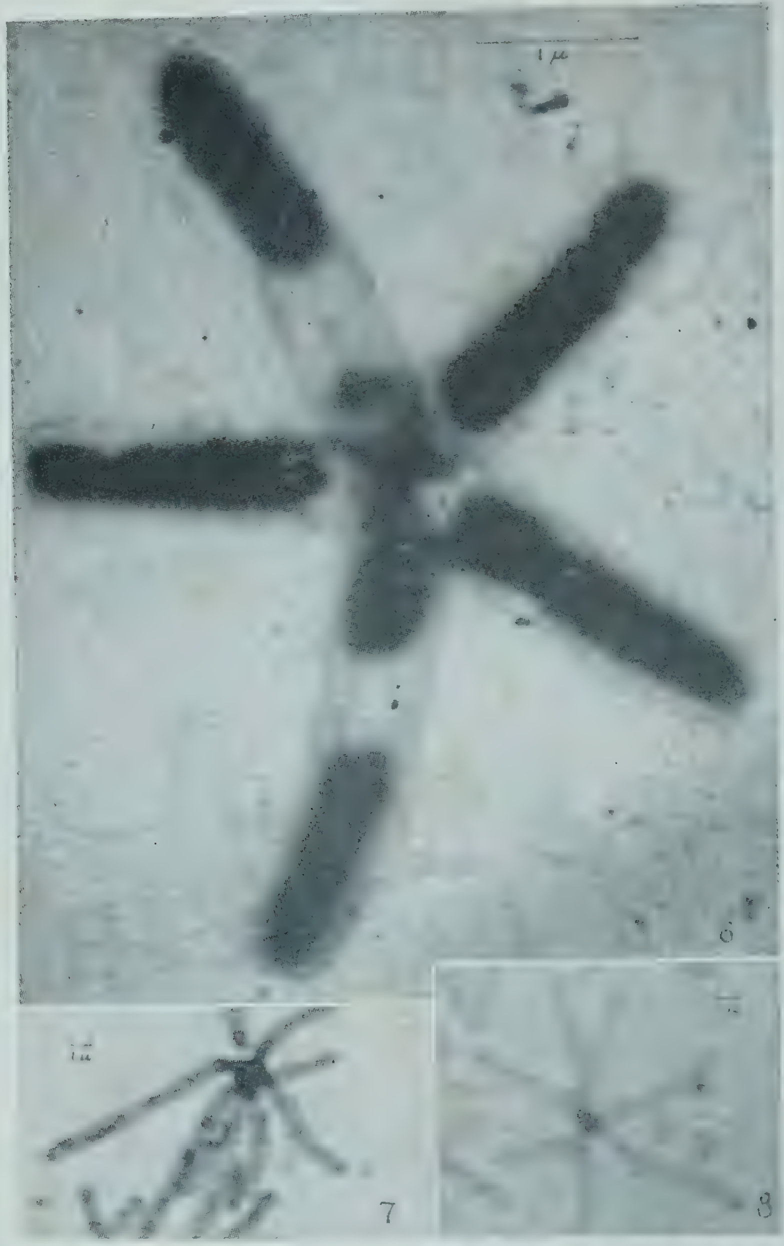

Fig. 338. *Agrobacterium tumefaciens*. No. 6. Electron micrograph of a 4-day bacterial star. Only a moderate elongation of the cells has resulted in this specimen. Note that the central mass has approximately the same opacity as the bacterial protoplasm. × 16,875. (Photograph by J. A. Carlile.) No. 7. Light micrograph showing chromatinic material. The preparation was hydrolyzed in N/1 HCl at 58° C. for 30 minutes. Loeffler's alkaline methylene blue stain was used. A concentration of chromatinic material is found at the center of the aggregate. × 988. (Photograph by J. A. Carlile.) No. 8. Light micrograph showing a single, well-defined, Feulgen-positive body at the center of the bacterial star. The preparation was hydrolyzed at 58° C. for 35 minutes and stained according to the Feulgen procedure. × 988. (Photograph by J. A. Carlile.)

TABLE XVI

SOME CHARACTERISTICS OF SEVERAL SPECIES OF ORGANISMS ASSOCIATED WITH THE SOIL, PLANTS AND FECES

Organism	Gram Stain	Morphology	Motility	Spores	Oxygen Relationship	Favorable Temp. Range (°C.)	Usual Habitat	Growth on Simple Media with Organic C	H_2S	Reduce NO_3	Indol	Proteolysis	Utilization of Carbohydrates (*glucose*)		Gas Producing	Fix Free N	Relation to Plants
													Acid	CO_2			
Rhizobium meliloti....	−	Rod	+	−	Aerobic	20–30	Soil	+	+	+	..	−	+	−	−	+	Produce root nodules
Agrobacterium tumefaciens	−	Rod	+	−	Facult.	20–30	Soil	+	..	+	+	−	+	−	−	−	Produce plant galls or nodular growth
Agrobacterium radiobacter	−	Rod	+	−	Aerobic	20–30	Soil	+	+	+	..	−	−	−	−	−	Found on roots No invasion
Aerobacter cloacae....	−	Rod	+	−	Facult.	25–40	Soil Intestine	+	−	+	−	+	+	+	+	−	None known
Serratia marcescens...	−	Rod	+	−	Facult.	25–30	Soil	+	+	+	−	+	+	+	+	−	None known
Erwinia carotovora....	−	Rod	+	−	Facult.	25–35	Soil	+	−	+	−	+	+	+	+	−	Plant pathogen Produces rots
Escherichia coli.......	−	Rod	+	−	Facult.	30–45	Intestine	+	−	+	+	−	+	+	+	−	None known
Pseudomonas fluorescens..............	−	Rod	+	−	Strict Aerobe	20–30	Soil Water	+	..	+	−	+	+	−	−	−	Pathogen of plants
Alcaligenes faecalis....	−	Rod	+	−	Facult.	30–40	Soil Intestine	+	..	+	−	−	−	−	−	−	None known

leguminous or other plants with which it is commonly found in contact. Some of the differences and resemblances between *Rhizobium, A. radiobacter* and *Aerobacter aerogenes* and similar species are seen in the accompanying table (Table XVI).

Some very suggestive relations between plant neoplasms (tumors) and tumor-producing bacteria have been revealed by White at the Rockefeller Institute. Strains of the microorganisms cultivated in media containing glycine lose the power to produce plant tumors, but if the plant tissues are treated with certain growth-stimulating substances (hormones), tumors develop when the bacteria are present, although neither bacteria nor hormone alone produce the neoplasms. In another series of experiments certain plants were inoculated with the organisms and then held for ten days at about 46° C. The bacteria seemed to be destroyed and no tumors developed at this high temperature. However, tumors developed when temperatures were lowered to normal, suggesting that the microorganisms had so altered the plant cells that they acquired the property of tumor formation in the absence of the living bacteria.[10]

However, much more on this subject may be learned from studies on the reactions of plant cells in tissue cultures to various agents; for example, extracts or products of bacteria, etc.[10a]

Agrobacterium tumefaciens and several related organisms exhibit a very curious growth phase, referred to as "star formation," in certain fluid culture media. In the film of growth on the fluid surface the bacteria aggregate in stellate formations, each rod with one end at the center. The mechanism and significance of this is not clear. Braun and Elrod have shown that it is not due to entangling of flagella or to attraction of several organisms "head-on" to a common central foreign particle. By means of the electron microscope and Feulgen staining they have indicated that there may be nuclear fusions between the organisms of a group, suggestive of a simple form of sexuality, but drew no final conclusion on this point.[11]

Transmission of Plant Diseases. The experimental plant pathologist accomplishes the infection of healthy plants with pure cultures of pathogenic bacteria by spraying the plant with a suspension of the desired organism or by pricking the plant, stem, root, leaf or seed with a needle carrying the bacteria in question. In nature, these operations are accomplished by wind and water carrying microorganisms, infected dust, pollen or parts of plants from one place to another. The bite or sting of insects which have just been feeding on diseased plants infects healthy ones. The bite of insects is also a means of transmission not uncommon in human disease as we shall point out later (yellow fever, malaria, bubonic plague, Rocky Mountain spotted fever). A farmer or horticulturist may, like a surgeon, also transfer pathogenic bacteria on pruning or other instruments unless he is careful to disinfect them. He may leave stumps of amputated branches open to infection unless he covers or disinfects them. Good paint, cresol, or bichloride solutions are useful for this purpose. The grafting of diseased buds or stems onto healthy ones may infect the host, or vice versa.

An interesting use has been found for streptomycin in freeing budwood, used for grafting valuable plum varieties, of the destructive *Xanthomonas pruni* which causes a black spot and canker in the new tree.[12]

Control of Plant Diseases. No universal rule can be laid down for the control of all plant disease; neither can we control all human or animal disease by any single procedure. However, some general precautions may be taken which may reduce the amount of damage and the spread of plant infections. Diseased plants or portions of them should be burned. In handling them, the utmost precaution must be taken not to allow them to come into contact with other susceptible plants. The soil around them may be disinfected if it is of small extent, or it may be allowed to remain fallow for a year or more. Instruments and gloves used in handling diseased plants should be disinfected.

Control of insect pests will reduce the transmission of many plant diseases. Eradication of weeds also may reduce disease, since these serve sometimes to harbor not only transmitting insects, but the disease organisms themselves. Analogous relations exist between many lower animals and man. Animals serve as "reservoirs" of many human pathogens, such as *Salmonella* (rats, mice), *Brucella* (cattle, swine), and so on.

Spraying plants with Bordeaux mixture or lime-sulfur early in the season and at ten- to fifteen-day intervals during the summer often prevents the ravages of disease, especially fire-blight. Copper sulfate (the effective agent in Bordeaux mixture) and sulfur are effective general fungicides. These do not always *cure* the diseases, but usually prevent their development. Spraying, therefore, should be regarded as a *prophylactic* or *preventive* measure. Spraying is especially desirable during hot, damp weather.

Finally, as with human beings, sturdy, well-nourished and well-watered plants are more resistant to all sorts of parasites. Certain individual plants sometimes stand out unharmed in a whole field ruined by some disease. Such plants should be cared for and their seed preserved. In such manner naturally resistant varieties are propagated and often net their discoverers a great deal of satisfaction if not money.

An important and widely used means of preventing the spread of plant diseases from one field to another is the disinfection of seeds, bulbs or cuttings used for propagation. This is done by soaking them for short periods (2 to 6 hours) in solutions of disinfectants such as bichloride of mercury, or exposing them to formaldehyde vapors under a tarpaulin. The procedures vary for different crops and details will be found in the books on agriculture and plant pathology.[13–17]

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Section IV

SOME PATHOGENIC MICROORGANISMS

INTRODUCTION

The distinction between pathogenic and nonpathogenic is at best a hazy one. Many microorganisms have never been associated with any pathological condition. There are many nonpathogens, such as autotrophs of the soil. Others are always found associated with disease and never otherwise. These are regular pathogens. Examples are the organisms of gonorrhea, measles and whooping cough. Between these definite groups of pathogens and nonpathogens is a long list of organisms of illy defined status (occasional pathogens, opportunists, etc.), some found in disease processes only rarely, or only in mild conditions, others found regularly in normal persons but capable of causing rapidly fatal disease, and so on through every phase of host-parasite relationship.

In this section we shall discuss representative organisms generally regarded as dangerous pathogens but sometimes occurring in carriers, and a few whose position as pathogens is debatable. Clinical discussions are reduced to a minimum.

Chapter 41

THE PATHOGENIC ENTEROBACTERIACEAE

We have dealt in an earlier chapter (Chapter 34) with enteric bacteria (Enterobacteriaceae) commonly found in soil, water, foods, the intestine and minor infections. Here we shall discuss the three genera of important pathogens in the Enterobacteriaceae, namely, *Salmonella, Paracolobactrum* and *Shigella*.*

It is of importance to note that these three genera differ from all of the previously discussed "coliform group" of organisms (*Escherichia coli,* etc., and *Aerobacter*) in (1) fermenting lactose and sucrose slowly or not at all and (2) in not digesting gelatin. Morphologically, and in many physiological respects, *Salmonella, Paracolobactrum* and *Shigella* closely resemble one another. They may be differentiated as shown in Table XVII. There is considerable immunological inter-

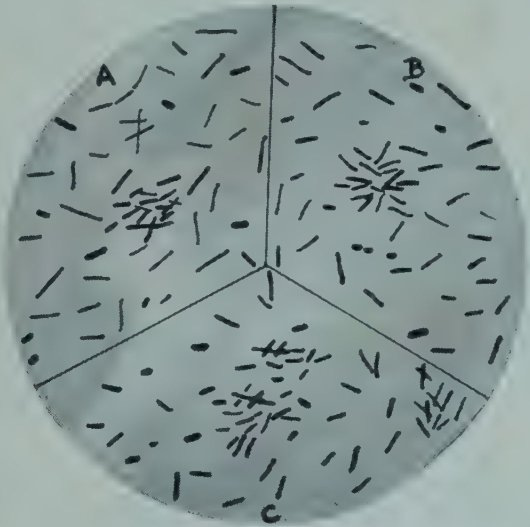

Fig. 339. Various members of the tribe Salmonelleae. *A, Salmonella choleraesuis; B. Salmonella typhi; C, Shigella paradysenteriae* (× 900).

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* The apparently meaningless generic names *Salmonella* and *Shigella* are derived from names of famous bacteriologists. Salmon was an American scientist, noted for his work on hog cholera (1885). Shiga was a Japanese bacteriologist who first determined the cause of highly fatal epidemics of dysentery in Japan in 1896.

relationship between some species of each genus and with the *Escherichia,* etc.

Various biochemical properties, such as ability to ferment various carbohydrates and production of hydrogen sulfide, serve to distinguish the species in each genus, as shown in Chart II.

Serological (agglutination) tests are used in addition.

TABLE XVII

DIFFERENTIATION OF PATHOGENIC ENTEROBACTERIACEAE

Genus	*Gas** from Dextrose	Motility	Indol
Salmonella	+	+	—
S. typhosa	—	+	±
Shigella	—	—	±
Paracolobactrum ...	+	+ or —	+ or —

* All ferment dextrose with *acid* formation.

Organisms are often found in which these differential tests give typical or inconclusive results, such as indologenic or anaerogenic (non-gasforming in glucose) or non-motile strains of *Salmonella.* Further, antigenic relationships (cross agglutination or possession of common antigenic fractions) have been described between *Salmonella, Shigella, Escherichia, Proteus* and other genera, so that it is frequently necessary to go through a complex process called antigenic analysis before the true status of an organism is known; even then the position of many is left in doubt. Some of these difficulties have been described by Saphra and Wassermann.[2]

An interesting possible means of studying relationships between the enterobacteriaceae is seen in the observation that various species produce substances which are antagonistic to others, *i. e.,* antibiotic substances. *Shigella* strains are most susceptible, *Escherichia,* next most susceptible, and *Paracolobactrum, Salmonella, Proteus* and *Aerobacter,* respectively, less susceptible. The method deserves further investigation.[1] For example, some strains of *E. coli* have been found to produce substances which are antagonistic to some dysentery bacilli (*Shigella*).[1a]

GENUS SALMONELLA

Of the salmonellas, *S. typhimurium, S. paratyphi* (*"paratyphosus A"*), *S. schottmülleri* (*"paratyphosus B"*), *S. enteritidis* and *S. choleraesuis* are among the most important. They, and some related species, constitute the "paratyphoid group."

The causative organism of typhoid fever, for years classed in a separate genus, *Eberthella,* named for Eberth, the first to describe the

CHART II

DIFFERENTIATION OF INTESTINAL SPECIES OF ENTEROBACTERIACEAE

- Dextrose ⊕
  - Gas always predominantly CO_2: Voges-Proskauer +; Methyl red −; Cellobiose ⊕; Citrate utilization tests +
    - motile; gelatin +; not encapsulated: *Aerobacter cloacae*
    - nonmotile; gelatin −; encapsulated: *Aerobacter aerogenes*
  - Gas always predominantly H: Voges-Proskauer −; Methyl red +; Motile
    - citrate utilization tests +; indol −; cellobiose ⊕: *Esch. freundii* ("Citrobacter")
    - citrate utilization tests −; indol +; cellobiose −: *Esch. coli*

LACTOSE ⊕ NONPATHOGENS

LACTOSE − PATHOGENS

- Dextrose + or ⊕
  - Acid and gas formed from dextrose; all motile
    - Sucrose ⊕ gelatin +; *Proteus vulgaris*
    - Sucrose −
      - Xylose − *Salmonella paratyphi*: nitrates not reduced; H_2S not formed; inositol −
      - Xylose +
        - Sorbitol − *S. suipestifer* and *S. choleraesuis*: H_2S produced
        - Sorbitol +
          - Inositol − *S. enteritidis* and *S. hirschfeldii*: nitrates reduced; H_2S produced
          - Inositol + *S. schottmülleri* and *S. typhimurium*: nitrates not reduced; H_2S produced
  - Acid formed, but *no gas*, from dextrose
    - Motility − *Shigella*
      - Mannite − *S. dysenteriae*
      - Mannite + *S. paradysenteriae* and others
    - Motility + *Salmonella typhosa*: mannite +; nitrates reduced; H_2S produced

Note: + = The organism attacks, or produces a positive test, or produces a fermentation without gas. ⊕ = Fermentation with gas. − = Negative reaction.

organism in 1880, is now grouped with the salmonellas as *S. typhosa* because of its immunological relationships.

Antigenic Analysis. The use of the agglutination test for the analysis of antigenic structure and classification is beautifully illustrated by the genus *Salmonella*. As stated in a previous chapter (see page 315), many closely related species of bacteria may contain one or more antigenic components in common. It was explained that agglutinating sera prepared by the injection of animals with such species often show much cross-agglutination with closely related species. By the process of dilution or agglutinin adsorption these sera can be deprived of virtually all but one type of agglutinin (monovalent sera) and with a group of such sera, representing all of the antigenic components in the genus, one may test any given organism for the presence of different antigenic components and assign to it a "formula" expressing the antigenic complex of which it is composed. This sort of antigenic analysis has been developed in the genus *Salmonella* to a high degree by such workers as Kauffmann, White, Edwards, Mickle and others, and we may now state the antigenic formula for each species.[3, 4, 5, 6]

In diagnostic work it is sometimes convenient to prepare group sera representative of several related species. For example, in one useful system group, Group A, serum agglutinates *S. paratyphi* and related species; Group B, *S. schottmülleri* var. Java and some other varieties; Group C serum, *S. choleraesuis* var. Kunzendorf, *S. amherstiana* and similar organisms, and so on (see Table XVIII). Individual specific sera may be used to differentiate related varieties in each group. The practical use of such a system has been described in detail by Kauffmann and Edwards,[6] Borman, Wheeler, Mickle, et al.[7]

Kauffmann-White Schema. By means of antigenic analysis the salmonellas have been arranged in a series often referred to as the "Kauffmann-White schema." In this schema the O (somatic) antigens are given Roman numbers and the flagellar or H antigens arabic numbers if of the group phase (Phase II). If in the specific phase (Phase I) the H. antigens are given small Roman letters.

The antigenic structure of any given species may therefore be expressed in terms of these numbers and letters. For example, *S. typhimurium* has the antigenic formula IV, V, XII; b. i; 1, 2, 3 (see Table XVIII).

The terms group phase and specific phase refer to variations in the antigenic structure which occur in the flagella of some species. Some of the organisms are said to be biphasic, that is, they may occur in both phases. It is possible to induce the appearance of, or to isolate, one or the other phase by cultivating one phase in soft agar containing antiserum for that phase. The antiserum will immobilize that phase and some of the organisms, changed to the other phase but not immobilized by the serum, will migrate outward in the soft agar and may thus be isolated. This is spoken of as the Gard technique. A useful modification employing a small U tube was described by Hajna.[7] Details of the complex phenomenon of phase variation have been discussed by Edwards and Bruner.[7a]

Many antigenic types of *Salmonella* very closely resembling the major species named above, differing perhaps in only one antigenic component, or one biochemical character, have been given species names. Some of these names are derived from the place where they were found, as, for example, *"senftenberg," "newport," "infantis,"*[8] *"mississippi."*[9] The list of species is long.[10] It is of interest to note that the antigenic structures of some of these species may be significantly altered, either by "artificial" means,[11] or as a result of natural processes.[12] Some of the antigenic types are completely interconvertible.[13] The significance of these changes for the utility of the Kauffmann-White system is still undetermined. One wonders whether such changes can in any way account for the new types which are constantly being described.[14, 15]

TABLE XVIII

ANTIGENIC FORMULAE OF SOME SALMONELLA SPECIES

Group	Species	O Antigens	H Antigens	
			Phase I	Phase II
A	S. paratyphi	I, II, XII	a	....
B	S. schottmülleri	(I) IV, V, XII	b	1, 2
	S. typhimurium		i	1, 2, 3
C	S. hirschfeldii	VI, VII	c	1, 4, 5
	S. choleraesuis		c	1, 3, 4, 5
D	S. enteritidis	IX, XII	g, o, m	....
	S. pullorum		....	....
	S. typhosa		d	....

Salmonella Infections. All of the Enterobacteriaceae are transmitted by feces or urine, or both, of patients or carriers. The most common mode of transmission of the salmonellas is by means of food which has become infected and allowed to stand in a warm place, after little or no cooking, so that the organisms can grow. These organisms grow well at warm room temperatures (80 to 100° F.). Infection of the food is sometimes from a very mild case or human carrier of *S. schottmülleri* or possibly by contamination with the excrement of mice or rats which harbor particularly *S. typhimurium* as well as other salmonellas. So-called "meat poisoning" results from eating raw or improperly cooked flesh of cattle, swine or other animals suffering from infection with these organisms, especially *S. choleraesuis* and *S. enteritidis.* These species are among the most commonly occurring in this country.[16] Animals of all sorts harbor a great variety of *Salmonella.*[17]

Obviously, avoidance of these diseases means cleanliness in the kitchen; sanitary habits on the part of food handlers; care to see that food is properly cooked to kill all organisms, even those in the center of large masses; proper refrigeration of stored food; and avoidance of uncooked foods at club suppers or on picnics, often prepared during the morning or previous evening and then unwittingly *incubated in the kitchen* or in transit. *Salmonella* food infections are very common. Samples of typical outbreaks are given in the bibliography.[18, 19]

The diseases caused by organisms of the *Salmonella* group are usually types of gastro-enteritis, prominent symptoms being fever, abdominal pain, nausea, vomiting, headache, diarrhea and general weakness coming on from ten to twenty-four hours after the infectious meal. Usually the outcome is not fatal but outbreaks differ in this respect. As shown by Seligmann, Saphra and Wassermann[16] many other conditions, as meningitis, pneumonia, etc., may be caused by *Salmonella* species.

Methods for laboratory diagnosis of these infections are similar in all respects to those used for typhoid fever (see page 730). Blood cultures are not so regularly successful, especially in the acute forms of salmonellosis, but should never be neglected. Spinal fluid cultures are not infrequently indicated, especially in children.

The Typhoid Bacillus (*Salmonella typhosa*). This organism is like the other salmonellas, paracolobactrum and shigellas in causing gastroenteritis. It differs in that it primarily invades the blood stream, and localizes in viscera, bone marrow, etc. Biochemical and other properties differentiating *S. typhosa* are shown in Chart II and Tables XVII and XVIII.

Typhoid Fever. Typhoid fever serves to illustrate a number of important bacteriological principles. The disease may be transmitted by any means which effect the transfer of infectious feces or urine to the mouth and so to the intestines of normal human beings. Of these means of transmission we must remember especially the four "F's": Feces, Fingers, Food and Flies. Water and milk are also notorious in this respect. The organisms, once ingested, are localized in lymph nodules (Peyer's patches) in the small intestine but soon invade the blood stream and may easily be cultivated from the blood during the first week of the disease. They establish themselves in bone marrow, spleen, etc. In about seven to ten days more, they cause the Peyer's patches to ulcerate and then get into the intestine and may be isolated from feces for periods of from a few days to months or years after recovery. After about ten days from first appearance of illness, antibodies appear in the blood and the bacilli cease to be found there. Blood culture diagnosis is no longer feasible. Agglutinins are found active against flagellar proteins of the bacilli: H agglutinins—specific for *S. typhosa* alone; and O agglutinins against the somatic antigens, some of which will also agglutinate certain other salmonellas which contain those same O antigens. Agglutinins also appear which are directed against an antigen having many properties of a capsule and which contributes to the virulence of the bacilli. Because of its relation to virulence it is called a "Vi" antigen. It engenders production of Vi agglutinins. Several other antibodies are also produced but are less fully understood than H, O and Vi agglutinins.

The H, O and Vi antibodies increase in amount until, by the second week, they are easily demonstrable in the blood serum by test tube methods and furnish valuable means of diagnosis.[20–24] At the same time protective antibodies appear, which can be demonstrated to protect mice against experimental infection with graded doses of virulent typhoid bacilli.[25, 26] These are not identical with H or O agglutinins but may be related to Vi agglutinins.

An interesting observation in this connection is that O typhoid agglutinins can be *prevented from acting* by coating typhoid bacilli with certain polysaccharides such as dextran, or pectin, or mucin.[27] When one remembers that the capsules of bacteria appear related to virulence or resistance to antibodies in the infected body, and that the capsules often consist of polysaccharides or mucin-like substances, the possible significance of this observation is clear. It is especially interesting that Vi antigen, probably a carbohydrate complex, also prevents the action of O agglutinins.

Carriers. If a patient survives the attack of typhoid fever, he may in a few instances continue to harbor the bacilli in some secluded retreat in the tissues or gallbladder for months or years, yet appear perfectly healthy. This is a good example of mutual adaptation. The organisms are often excreted regularly or intermittently in the feces and the convalescent becomes a carrier and a menace to the health of others.[28, 29, 30] An excellent example is cited in the bibliography.[31]

After recovery, the blood begins to show diminution of agglutinins, especially of the Vi and O antibodies. H agglutinins tend to persist. If the patient becomes a carrier, his blood often continues to have a demonstrable titer of Vi agglutinins. The continued presence of Vi agglutinins in the blood seems to be directly dependent upon the actual presence in the body of typhoid bacilli. Detection of Vi agglutinins in the blood of an apparently healthy person, therefore, indicates that that person is a carrier.[20–24]

Typhoid Vaccination. Probably many of my readers will have received typhoid "shots" at some time before studying microbiology.

The process is a good example of a method active artificial immunization. The material usually injected is saline solution containing about one billion typhoid bacilli per ml., killed by heating at about 65° C. for 30 minutes or with formaldehyde. Usually included also are about the same number of killed *Salmonella schottmülleri* and one-half billion *S. paratyphi*.

As previously noted, in preparing a bacterin care must be taken to use organisms of the greatest immunogenic value. In the case of *S. typhosa* this means smooth, motile strains of high Vi content and highly virulent. Other variants, particularly R strains, may have little or no protective value.[32] That Vi substance is of importance in producing immunity to typhoid is shown by the fact that Vi antigen removed from certain *Escherichia* is an effective immunogen.[33]

The value of immunization is well shown by the experience of exposed persons in an outbreak already referred to. That protection is not absolute, however, is shown by the occurrence of typhoid in immunized persons.[34, 35] Much depends on individual susceptibility and dosage of infection received.

For initial immunization, 3 doses (0.5 ml., 1.0 ml. and 1.0 ml.) at weekly intervals are required. Smaller doses are not so effective. Revaccination with small doses (0.1 ml. intracutaneously or 0.5 ml. subcutaneously) annually is recommended in order to maintain immunity at an effective level.[36, 37] This is a good example of the use of the secondary antigenic stimulus (see page 330).

These facts concerning typhoid vaccination are worth noting in detail because they illustrate principles of basic importance in relation to most other bacterial vaccines and also to the use of antigens like diphtheria toxoid.

GENUS SHIGELLA AND BACILLARY DYSENTERY

The shigellas (also commonly known as dysentery bacilli), cause intestinal disturbances ranging from very mild diarrhea to severe dysentery with intense inflammation and ulceration of the large bowel, often with scar formation and stricture of the bowel after recovery (*bacillary dysentery*).[38] In some epidemics of bacillary dysentery, especially those due to *S. dysenteriae*, the fatality rate is high. The organisms do not usually invade the blood stream.

The genus contains a number of species and many antigenic types, in this respect resembling the salmonellas.[39-45] The principal species of *Shigella* are listed in Table XIX, with differential tests. In this country *S. dysenteriae* is rarely found in carriers but has occurred in some epidemics. Other species, listed below, often cause sporadic and epidemic infections in this country and elsewhere and are commonly found in healthy persons everywhere.[39] Several closely allied organisms cause similar conditions. These may be distinguished from the very toxic *S. dysenteriae* by the characters shown in Table XIX. Fermentation tests are of limited value in identification of shigellas because the characters vary. Details of technical procedure are well summarized by Ewing and Bruner.[45a]

In Table XIX attention is called to the relationship between reactions on mannite and reduction of trimethylamine oxide.[46] The mannite-negative *Shigella* organisms are usually very pathogenic, antigenically homogeneous, and are culturally related. They are also uncommon in this country. The mannite-positive group is antigenically very heterogeneous, of relatively less pathogenicity, and common everywhere.

TABLE XIX

SOME CULTURAL REACTIONS OF THE SHIGELLAS

Species	Lactose	Xylose	Mannite	Maltose	Sucrose	Indol Production	Reduction of T.M.A.* oxide
Shigella dysenteriae	–	–	–	–	–	–	–
Shigella ambigua	–	–	–	–	–	+	–
Shigella paradysenteriae	–	–	+	±	±	±	–
Shigella sonnei	Slowly +	–	+	+	+	–	+
Shigella madampensis	Slowly +	–	+	+	+	+	+
Shigella alkalescens	–	+	+	+	–	+	+

* Trimethylamine.

Antigenic Relations of Shigella. Among the shigellas the situation is almost as complex as among the salmonellas. Only O antigens are dealt with since the shigellas are non-motile. This antigenic heterogeneity is found particularly in *S. paradysenteriae* and *S. alkalescens* species. *S. dysenteriae, S. ambigua* and *S. sonnei* are fairly homogeneous antigenically.

Various systems of grouping and naming have been proposed for the paradysentery group, the simplest being to give each antigenic type a Roman numeral.[40] As in the salmonellas, new types are frequently described.[41] The situation in the alkalescens group had been described by Neter.[42] That the situation as a whole is not simple is shown by the discovery of a *motile* organism related to *S. sonnei*.[43]

Dysentery. The transmission and prevention of bacillary dysentery* are similar to these aspects of typhoid fever and salmonellosis, except

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* As differentiated from amebic dysentery.

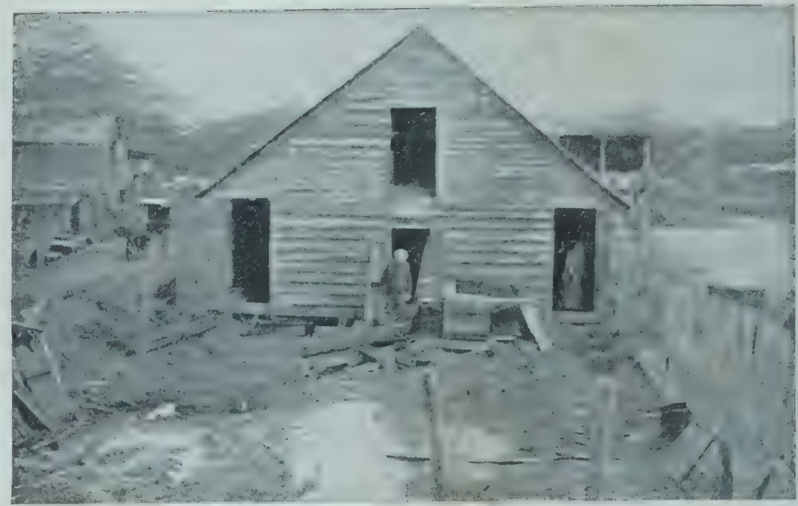

Fig. 340. Flies abound here. Unscreened privies and dwelling houses are close by in the background. The intermingling of human and bovine dung, contaminating food and milk, is fairly complete. Milk easily becomes contaminated from the feces in the privies via the flies. (Hygienic Laboratory Bulletin 56.)

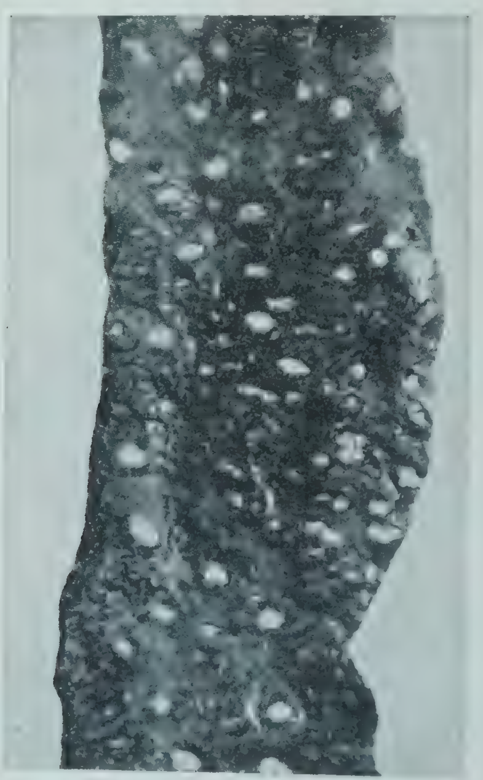

Fig. 341. Intestine of a child who died of bacillary dysentery. Ulcerated Peyer's patches are seen as whitish areas all over the lining of the intestine. (After A. Castellano.)

that animals do not commonly transmit typhoid and dysentery. Dysentery sometimes occurs in water-borne outbreaks, but milk is a particularly favorable medium for the shigellas. Two important means of transmission, perhaps the most important, are (1) direct transfer of feces via hands to other hands or to food, etc., and (2) by flies (Fig. 340). The "Four F's" are in evidence. Infants are frequently infected if an adult carrier (which is far from rare) unwittingly contaminates the baby's milk and then leaves it in a warm kitchen to incubate. This is the usual situation in "summer complaint" (Fig. 341).[39, 47] Carriers of dysentery are common. The use of sulfonamide drugs has proved useful in curing them.[48, 49]

Dysentery Vaccination. Vaccination against bacillary dysentery has long been studied but appears to be less satisfactory than typhoid vaccination.

An experimental study in which immunized human volunteers submitted to attempts at infection with *virulent* dysentery organisms (for mice) revealed facts of fundamental importance. After what appeared adequate injections of two kinds of bacterin, the volunteers received very large doses of live dysentery bacilli in milk and water; along with paregoric to produce an intestinal stasis. Other conditions favoring infection were devised and used, such as putting infective feces in capsules so as to protect the organisms from the acidity of the gastric contents.

It was found that (1) strains virulent for mice were often of low virulence for man and vice versa; (2) while the bacterins stimulated the production of immunity in *mice,* and protective antibodies (for mice) in *man,* they did not produce *significant immunity* in man; (3) even with the precautions used to insure infection, infection of unimmunized subjects was very irregular. These findings show that (1) immunological phenomena and resistance to dysentery are different in mice and men (hence the danger of generalizing for human beings from animal experiments); (2) the presence of antibodies circulating in the blood stream does not necessarily prove the presence of resistance to infection, especially when the infection is within the bowel or other organ or tissue not bathed richly by the blood stream; (3) resistance may be present in the absence of vaccination and dependent on obscure factors other than serum antibodies.[50]

GENUS PARACOLOBACTRUM

This group of bacteria is sometimes called "paracolon organisms" and may be regarded as occupying an intermediate position between the mainly saprophytic *Escherichia-Aerobacter* genera, and the parasitic *Salmonella-Shigella* group. Many workers have referred to the paracolon organisms as "aberrant coliforms." They have most of the cultural properties of the coli-aerogenes organisms, and some of the pathogenicity (probably) and antigenic structure, as well as some cultural properties, of the paratyphoid-dysentery group. The most distinctive property of the paracolons is inability to ferment lactose at all, or to do so promptly (within forty-eight hours). When colonies of these organisms are seen on plates of the selective dye-lactose media described later in this chapter, they are colorless and are often mistaken for organisms of the group of definitely nonlactose-fermenting pathogens.

One species of paracolon organisms (*Paracolobactrum aerogenoides*) is related to *Aerobacter;* another (*P. coliforme*) to *Escherichia coli;* and another (*P. intermedium*) to *E. freundii,* based on I M Vi C relations and antigenic structure. Those related to *Aerobacter* closely resemble the members of that genus but ferment lactose slowly or not at all. A similar relationship exists between the *freundii*-like paracolons and *E. freundii,* and so on. As shown by Stuart, Wheeler, Borman, Rustigian and others, the paracolon group is large and culturally and serologically complex.[51, 52, 53] There are many organisms in the group which do not agree exactly with the descriptions of named species, either culturally or serologically, and whose position is not clear. The group grades into the *Salmonella-Shigella* group on one end and into the coliform group at the other. Paracolon organisms are frequently found in food-infection outbreaks and are often mistaken for *Salmonella* or *Shigella* unless very complete cultural and antigenic studies are made of them[54, 55, 56] and unless lactose fermentation tests are incubated for at least a week.

The paracolons are widely distributed. Some very interesting serological relationships were found among salmonellas and paracolons occurring in reptiles, turkeys and man.[57] Similar relations have been reported by other workers, and a new, distinctive group of paracolons, the "Arizona group," is now recognized as common in reptiles and birds.[58, 59, 60]

A useful differential test for paracolons is based on urea decomposition. *Paracolobactrum aerogenoides* and *P. intermedium* as well as *Proteus* are positive, while *P. coliform,* salmonellas and shigellas are negative. The relationship to *Proteus* is of especial interest.[61]

LABORATORY DIAGNOSIS OF BACTERIAL ENTERIC INFECTIONS

Blood cultures. In these diseases invasion of the blood stream sometimes occurs. It regularly takes place in the early stages of typhoid fever, less commonly in *Salmonella* and rarely in *Shigella* and paracolon infections. Accordingly, in typhoid fever, laboratory diagnosis during the first four to ten days after onset depends on blood cultures. About 5 ml. of blood are drawn from a vein and transferred to a flask containing 100 ml. of 2 per cent bile broth. If desired, the blood may be allowed to clot and the serum used to test for

H, O and Vi agglutinins, although these appear in greater concentration later (after ten days). The clot will enmesh any bacilli, along with the blood cells, and may be macerated in 100 ml. of infusion bile broth. After incubation of the broth for twenty-four hrs. at 37° C. selective agar plates are streaked for isolation in pure culture as described below.

Cultures from Feces and Urine. When isolation of *S. typhi* from the feces is attempted, a bit (1 gm.) of the fresh specimen may be placed in a tube of fluid medium containing *sodium selenite,** and tightly stoppered. It is incubated for about fourteen hours. The selenite partly inhibits growth of the large numbers of *Escherichia* and other organisms present in feces but permits *S. typhi* to multiply. This method is less useful for *Salmonella* and is of still less value for *Shigella* since the selenite is toxic for some species. No data are available regarding paracolons.

After appropriate incubation of the selenite medium, (or broth blood cultures) plates of appropriate selective medium, (S-S† or desoxycholate*) are streaked and incubated for eighteen to twenty-four hours. These cultures often yield results when plates inoculated with the fresh stool specimen fail to yield the desired organism. A sample of the fresh stool is also streaked on the surface of the agar media.

These media contain (1) a nutrient base; (2) lactose; (3) an acid indicator like neutral red; (4) a substance inhibitory for most bacteria feces but not for pathogenic enterobacteriaceae. The colonies of nonlactose fermenting organisms (including paracolons) on these media are colorless or gray and translucent, round, and glistening. Colonies of other organisms either do not appear, because of the inhibitory action of the media, or are usually easily distinguished from those of the specific pathogens by their appearance. This is true especially if they be lactose-fermenting organisms, *E. coli,* etc., since their colonies turn red because of the effect of the acidity of the colony (from fermentation of lactose in the medium) on the neutral red.

If isolation of the pathogens from urine is to be attempted the urine specimen (15 ml.) is centrifuged for 30 minutes at high speed, the supernatant is poured off and cultures are made from the sediment onto media as described above.

Suspicious colonies on any of the plates are fished with a needle to tubes of sterile broth or nutrient agar as pure cultures. Once isolated in pure culture from blood culture or from feces by means of selective cultivation as described above, the organisms are subjected to biological studies including determination of their distinctive metabolic properties (see accompanying tables) and their agglutination reactions.

Serological Diagnosis. For the serological study of enteric diseases the method of receptor analysis is most reliable.[62] An increase in O agglutinins in the patient's serum from an initially low titer to a definitely higher titer as the disease progresses is especially significant (see section on immunological methods, page 323).

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† Available from Digestive Ferments Co., Detroit, Mich.

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Chapter 42

DIPLOCOCCUS PNEUMONIAE AND PATHOGENIC STREPTOCOCCI

GENUS DIPLOCOCCUS

British writers refer to *Diplococcus pneumoniae* as *Streptococcus pneumoniae*. Not only do the "pneumococci" often form chains (usually made up of from two to eight *pairs* of cocci) but, like some of the hemolytic streptococci, they are encapsulated. In blood-agar plates they produce alpha type zones. They are facultative, heterotrophic and parasitic. Methods of cultivation and study are like those used for streptococci. The exact nutrient requirements of pneumococci are complex; they include hydrolyzed casein, adenine, guanine, uracil (constituents of nucleic acids) and several vitamins, as well as minerals and organic carbon as glucose.[1] They also require carbon as CO_2. In organisms requiring both forms of carbon, the CO_2 is probably used in cell synthesis, the organic C compounds as sources of energy. *Diplococcus pneumoniae* is the most important species in this genus. It is found in the saliva and sputum of patients with *lobar pneumonia,* and like streptococci, also occurs frequently in the normal mouth and throat.

Distinguishing Characters of the Pneumococci.[2] The cocci are rarely spherical, having the form of short projectiles placed base to base. They are gram-positive. Pneumococci are *extremely pathogenic for white mice* when freshly isolated from the body. Advantage is often taken of this fact to isolate pneumococci from sputum of patients for diagnosis. The sputum is injected into the mice intraperitoneally. After six to twenty-four hours the mice die or become very ill, and enormous numbers of pneumococci are found in the peritoneal cavity and heart blood. Most or all of the other bacteria normally in the sputum are taken up by leukocytes or otherwise destroyed (Fig. 342). The heart blood or peritoneal exudate of the mouse may be streaked on infusion-blood-agar plates and *Diplococcus pneumoniae* isolated in pure culture. The colonies are thin, flat, translucent, greenish and from 0.5 to 2 mm. in diameter.

The cocci found on the peritoneum of the mouse may be identified tentatively by (1) their morphology; (2) their *capsules* (Fig. 343) containing carbohydrate which react specifically with antisera prepared by injecting animals with them; (3) their *solubility* in bile or solutions of various surface-tension reducents, of which bile is one*; (4) ability to ferment inulin (see Table XII). Pneumococci are the only streptococcus-like organisms, highly pathogenic for mice, bile soluble, which *ferment inulin* and produce the alpha type of appearance in blood-agar plates.

Serological Types of Pneumococci. A long study of pneumococci has shown that they may be divided into thirty-two or more serological types which are designated with Roman numerals.[3] These are analogous to the Lancefield groups of streptococci and, as in the streptococci, serological specificity is conferred by a carbohydrate. Immune sera are available representing each type. Pneumococci do not possess a type-specific M substance, like that of streptococcus of the beta type, as far as is known.

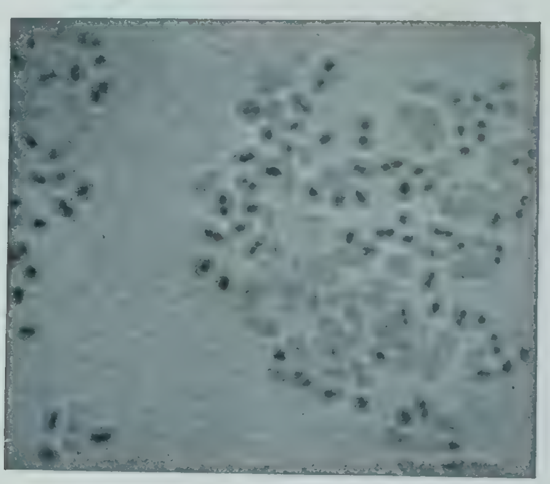

Fig. 342. Pneumococci with capsules in a smear preparation from peritoneal exudate stained by W. H. Smith's method; × 1500. The capsules are swollen by contact with type specific serum. Cf. Fig. 343. (W. H. Smith; photo by L. S. Brown.)

If the peritoneal exudate from a mouse, obtained as described above, or the fresh sputum from a patient with pneumonia, is mixed with each of these thirty-two sera, in separate tubes, or in droplets on microscope slides, *one* tube or slide preparation very often shows, after a few minutes, characteristic reactions including *agglutination*

* To determine this property, the peritoneum of the mouse is well washed by means of a fine-tipped pipette and small rubber bulb, with 1 or 2 ml. of salt solution and the washings collected in a small tube. After the mucus and leukocytes have settled out, the supernatant fluid, turbid with pneumococci, is transferred to another tube and about one-fourth of its volume of ox-bile (or, far better, 2 per cent solution of sodium lauryl sulfate) added. In a few minutes the fluid will become clear, due to the fact that the pneumococci are entirely dissolved. Broth cultures will exhibit the same phenomenon.

of the cocci, *precipitation* of polysaccharide derived from the capsules, and *swelling of capsules* (Figs. 342, 343). This swelling of the capsules is spoken of as a *"quellung"* (German for *swelling*) reaction. It was first described by Neufeld and is sometimes called the Neufeld reaction. Thus, we may say that the patient is infected with type I, II, III or some other type of pneumococci, according to type of serum which causes these reactions.

The procedure of determining the serological type to which pneumococci belong is often spoken of in the laboratory as "typing."[4, 5]

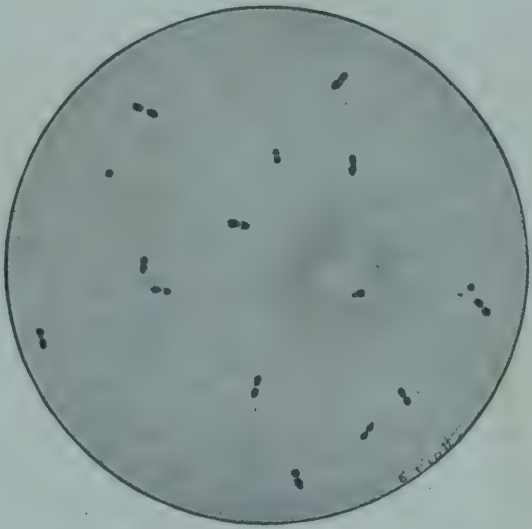

Fig. 343. Appearance of negative quellung reaction showing capsules not swollen. (From Lord and Heffron, "Pneumonia and Serum Therapy." Courtesy of The Commonwealth Fund.)

The "typing" of pneumococci is of therapeutic importance because an efficient antiserum exists for type I infections, and for some other types as well, especially II, IV, V, VII and VIII.[6, 7] However, since the advent of sulfonamide drugs and penicillin it is seldom now done as a routine procedure since these drugs act upon all pneumococci regardless of type.

Capsular Polysaccharides of Pneumococci. Type specificity of pneumococci depends on the presence, in their capsules, of a carbohydrate-protein complex which can be extracted by appropriate solvents and which is characteristic for each type. If deprived of their capsules, as when they vary toward the rough phase, they are *immunologically indistinguishable*. They are also *avirulent*.

These facts relating to the occurrence of specific carbohydrate substances in or on cells of bacteria are of much wider than medical interest because they illustrate a phenomenon of fundamental importance which is encountered again and again in microbiology—the occurrence among members of a species, of immunological "types" or groups which differ because of the chemical differences in their carbohydrates. The beta-type hemolytic streptococci described in a previous chapter furnish one example, the influenza bacilli another (see pages 547 and 780). Infection with each specific serological type of each species of organism is a separate disease, and the immunity it confers, if any, is only against that particular type and not against others, even of the same species.

Relations of Capsules to Virulence. Virulence of pneumococci is definitely associated with their capsules. In general, encapsulated organisms (if they have invasive powers) seem to be less readily taken up by leukocytes and also to be protected from the action of antibodies. The capsular substance also appears to be toxic *per se* as in type B influenza bacilli and pneumococci. Type III pneumococci have particularly heavy, mucoid capsules and were formerly known as *Streptococcus mucosus* or *Pneumococcus mucosus*. Probably because of their heavy capsules, type III pneumococci produce a particularly dangerous form of pneumonia, once they gain a foothold. A striking parallelism in this respect is seen between pneumococci and influenza bacilli of type B, as well as anthrax bacilli and *Clostridium perfringens.*

Transformation of Pneumococcal Types. A very interesting agent capable of inducing a permanent alteration in a bacterial cell is a desoxyribonucleic acid fraction extracted chemically from type III pneumococci. This has been described in Chapter 8.

Pathogenic Action of Pneumococci. Pneumococci are highly invasive organisms and, like beta hemolytic streptococci, can infect many different parts of the body and, according to their localization cause meningitis, septicemia, peritonitis, empyema, sinusitis, etc. The name pneumococcus is, of course, derived from the fact that they are frequently seen as the cause of pneumonia.

The method of transmission of pneumonia is chiefly through droplets of infected saliva and nasal and pulmonary mucus, and by inhalation of infected dust. Kissing undoubtedly transmits the infection but obviously not every such infection results in disease. Romance has a powerful ally in natural resistance to infectious disease. Pneumococci survive desiccation very well, and thus are found in dust in patients' rooms, hospital wards, etc.[8, 9]

The organisms are present in almost everyone's nose and mouth, but in order to produce disease appear to be obliged to await a reduction of the resistance of their victim by some other means, especially previous disease such as influenza, whooping cough or measles, or exposure to cold, starvation or great fatigue. Much fatal pneumonia occurs in very young children following diseases of childhood. Avoidance of exposure to such diseases in infancy (or under six years of age) is imperative. For adults, *especially elderly persons,* avoidance of contact with pneumonia patients and of excessive exposure and fatigue are important. By the use of type specific polysaccharides, active artificial immunity may be produced.[10]

BETA-TYPE STREPTOCOCCI OF GROUP A

Pathogenic Action. Among the commonest diseases caused by beta type streptococci of group A are infections of the tonsils and throat, sinuses, lungs and blood. Two representative clinical manifestations of throat infection are scarlet fever and septic sore throat.

Scarlet Fever and Septic Sore Throat. The organisms gain entrance to the throat by direct contact with a patient, or infected dust, milk, or some other means of transmission, find conditions in the tonsils or in the adjacent tissues favorable for their growth, and proceed to establish themselves, giving rise to the diseases mentioned by giving off toxins or invading the tissues and blood stream or both.

Any one of a number of cultural varieties or M types of group A streptococci may cause either disease. As in pneumonia, there is thus a certain lack of specificity as to etiology. Septic sore throat is the same disease as scarlet fever, differing only in the absence of a rash.[11] The rash in scarlet fever is due to the susceptibility of the patient to the *exotoxin* (*erythrogenic* or *rash-producing toxin*) of these organisms which is not type specific. Persons who have developed an immunity to this exotoxin may still contract *infection* with streptococci of any M type but *do not develop the rash.*

The Dick Test. The Dick test, devised by the Drs. Dick of Chicago, is performed by injecting into the skin a very tiny amount of group A streptococcus erythrogenic toxin. If, in about twenty-four to thirty-six hours, a red spot at least 1 cm. in diameter appears at the site of injection, it is inferred that the person has not sufficient antitoxin in his blood to protect him against even a minute dose of this particular toxin and is therefore likely to develop a rash (scarlet fever) when infected with strongly toxigenic strains. A similar test, called the Schick test, is used in diphtheria and is discussed in connection with the diphtheria bacilli (see page 743).

Other Pathogenic Actions of Hemolytic Streptococci. Hemolytic streptococci of Group A (also groups B, C, F and G) are associated with many acute, inflammatory, pyogenic conditions: meningitis, empyema, peritonitis, "blood poisoning" (streptococcemia), puerperal sepsis ("child-bed fever"), etc. The number of leukocytes in the blood stream increases greatly during such infections, although *local* aggregations of them may not occur as in *Staphylococcus* infections. Like the staphylococci, all group A beta-type streptococci produce hemotoxin, leukocidin, exotoxin and endotoxin. Streptococci of groups A, C and G produce an enzyme (fibrinolysin) capable of dissolving human blood clot (fibrin) as well as a trypsin-like enzyme (histase) digesting dead tissues.[12, 13] It is likely that these enzymes interfere with or neutralize defensive measures of the human host and permit the cocci to invade tissues and blood vessels more rapidly.

One of the most important causes of disability and death, rheumatic fever, is probably caused by allergic complications following infection with hemolytic streptococci of group A. The exact role of the streptococci is not clear but epidemiological studies indicate their involvement in the disease.[14]

Immunological Reactions. Infection of human beings with hemolytic streptococci of group A usually stimulates the formation of several different kinds of antibody. Examples of these are protective substances, agglutinins, precipitins, antihemolysin, antifibrinolysin, etc.

PATHOGENIC ACTION OF ALPHA-TYPE STREPTOCOCCI

Although constantly present in the normal mouth, usually without deleterious effects, these organisms can at times gain a foothold in the body. Hemolytic streptococci of the alpha type tend to cause chronic, but none the less dangerous, diseases. They are frequently found in abscessed teeth and diseased tonsils and are always present in saliva. From teeth and tonsils they may be carried to the joints and produce rheumatic conditions. They can also infect the heart valves causing a serious heart disease, *bacterial endocarditis.*

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Chapter 43

DIPHTHERIA, TUBERCULOSIS, LEPROSY

DIPHTHERIA[1]

Diphtheria is a very specific disease due to only one species of organism, *Corynebacterium diphtheriae*. The organisms are transmitted in the same manner as others causing respiratory diseases (see page 361). Healthy carriers are common (about one per cent of the population) and are doubtless sources of cases. The bacteria establish themselves on the mucous membrane of the throat and nose and proceed to excrete their exotoxin.

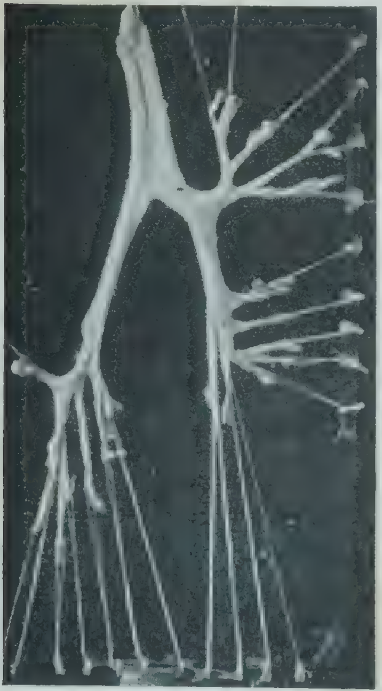

Fig. 344. Diphtheritic membrane removed after death showing extension into air passages of both lungs. (Tull. J.A.M.A., Vol. 104.)

Toxin Production by Corynebacterium Diphtheriae. *C. diphtheriae* secretes into its environment one of the most powerful biological poisons known. It has been shown that this toxin is either derived from, or closely associated with, one of the respiratory enzymes of *C. diphtheriae,* probably cytochrome.[2] When the organism grows on the tonsils this poison is absorbed by the blood and will damage heart, kidneys, adrenals and nerves and may cause death unless antibodies against the toxin are already present or are developed rapidly by the cells of the patient, or are injected into him from some outside source. The toxin is present in broth cultures of *C. diphtheriae* and can be separated from the bacilli by filtration. It is easily destroyed by heat, sunlight and oxygen and must therefore be kept in a dark icebox in sealed vessels. It is very poisonous to rabbits, mice, guinea pigs and to chicks and other birds, as well as to human beings when injected or when absorbed from infected tissues.

Corynebacterium diphtheriae is a classical illustration of the fact that virulence of a pathogenic organism may consist of either or both of two factors, *aggressiveness* and *toxigenicity*. This organism, although often growing extensively on the surfaces of the respiratory tract in the throat has little ability to invade the tissues beyond the mucous membrane. It is not, therefore, very aggressive. It produces death, however, because of its extreme toxigenicity (Fig. 344).

Fig. 345. Immunization to diphtheria. This picture shows the simplicity of the procedure. It is of great historial interest, since it is a portrait of the late Dr. William H. Park, long Bacteriologist for the City of New York, who introduced diphtheria immunization to this country (right); and Dr. Schick, the famous Austrian physician who devised the Schick test for immunity to diphtheria (standing, white gown).

Most persons above about 15 years of age possess a natural, *active* immunity probably through *subclinical* or unrecognized, mild attacks during earlier childhood, and retain their immunity throughout life, probably as a result of repeated reinfection. Their blood usually contains a small amount of antitoxin and this is sufficient to combat ordinary infection.

The Schick Test. It is possible to determine whether or not a person's blood contains sufficient antitoxin to protect him. The principle of the test is exactly similar to that of the Dick test for scarlet fever susceptibility. The test is made by determining ability to withstand a small dose of toxin. Of the amount of diphtheria toxin which takes four days to kill a small guinea pig (1 minimal lethal dose, as it is called), one-fiftieth is injected *into* the skin of the person whose immunity is to be tested. If the person's blood contains a sufficient amount of diphtheria antitoxin to protect him (about 0.01 unit* per cubic centimeter of serum), nothing happens. If his blood contains too little antitoxin, a small red spot appears at the site of the injection and remains for some days. This is called a positive *Schick test,* the test having been devised by the Austrian physician, Schick. It is perfectly harmless (Fig. 345) and may do some good by stimulating antibody production.

If the natural processes of often-repeated antigenic stimulation referred to above, which depend on prevalence of healthy carriers of virulent organisms, are diminished due to decreasing numbers of such carriers, then many persons may either fail to retain their childhood immunity or may not be naturally immunized in the first place.[3] Their Schick test may revert to positive. To supplement natural immunization processes artificial active immunization is widely used.

Active Immunization to Diphtheria. Children giving a positive Schick test may contract diphtheria or they may not; depending on dosage of infective bacilli, natural resistance and other factors. For safety, they should be artificially, actively immunized sufficiently so that they give a negative Schick test. Any physician or health department will do this on request. The process requires only two injections of a specially treated diphtheria toxin called *toxoid.* Inasmuch as this immunity wears away in time, it may be advisable to reimmunize with a single small dose after a few years. This is probably sufficient for life. Certain persons seem to remain susceptible in spite of apparently adequate immunization, and may contract the disease. There is evidence that an antibacterial as well as antitoxic immunity might help such persons.[4, 5]

Adults not infrequently give positive Schick tests, but do not have diphtheria nearly so often as children, from which it may be inferred that such persons have the ability to *respond to infection very rapidly* by producing antitoxin in their blood. This power is probably due to a previous slight infection or *primary stimulus* which has conditioned their tissues to make this rapid defensive response to a second stimulus.[6] It illustrates a fundamental principle in immunity (see page 330).

Natural vs. Artificial Immunization. While much diphtheritic disease and many deaths have doubtless been prevented by artificial immunization there is evidence that the immunity to this disease conferred by *actual infection,* subclinical or severe (natural immunization), is more effective and durable than that conferred by injections of toxoid. For example, among survivors of clinical diphtheria (unless treated by antitoxin) second attacks rarely occur, while cases of diphtheria of all grades of severity, including fatal ones, are reported not infrequently in children supposedly protected by toxoid injections.[7-11]

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* A unit, roughly, is sufficient antitoxin to neutralize a little over 100 minimal lethal doses of toxin.

This strongly suggests that something is lacking in the artificial immunizing agents (toxoids) commonly used. Modern diphtheria antigens are prepared from purified toxoids and the present tendency is still further to purify them.[12, 13, 14] However, the immunity evoked by such pure toxoids is presumably largely against the *exotoxin* of the diphtheria bacillus and, judging by many apparent failures to protect against infection and death, does not confer immunity against the living bacillus; that is, against its *endotoxins.* There is good evidence that these *endotoxins* are dangerous substances and that they are closely related to the *exotoxins.*[15] There is also good evidence that immunization against *endotoxins* alone is very effective in protecting against diphtheritic infection and against diphtheria exotoxin.[4, 5, 16] The protection given does not depend on antitoxin but on a local binding action of the tissues (see immunity in tuberculosis; also page 349).

These facts would suggest the advisability of including somatic antigens (endotoxins) of the bacilli as well as pure toxoids in materials injected for protection against diphtheria. The same basic considerations might apply in artificial immunization procedures such as prophylaxis against tetanus, scarlet fever and other infectious processes in which both exo- and endo-toxins may be involved.

Passive Immunization to Diphtheria. As pointed out in the section on immunity, a person ill with diphtheria, or a child exposed to the disease by living in the same house with such a patient, may have immediate need of antibodies to combat the disease or ward off infection. Artificial, active immunization with toxoid, as described above, is too slow. Three to six weeks is too long a time to wait for protection. Endangered persons may, under special circumstances to be judged by the physician, receive immediate protection through injections of serum which contains large quantities of antitoxin. This is passive immunity and is quite transitory. After the passive immunity has worn off (one to two weeks) or even while it lasts, *active* immunization should be used as described above.

Laboratory Methods for the Study of Diphtheria Carriers and Cases.[17] The detection of diphtheria bacilli in the throat or nose of diphtheria patients, convalescents and normal persons is obviously of the utmost importance in the diagnosis and control of the disease. For this reason, special means have been devised for finding and isolating the organisms. One of the simplest cultural procedures consists in rubbing a wooden applicator tipped with cotton over the infected or suspected tonsils and nasal mucosa, and then rubbing the swab over the surface of sterile, coagulated serum (Löffler's medium[*]) or sterile coagulated eggs (Pai's medium[†]). After 10 to 24 hours' incubation at 37° C. a methylene-blue-stained smear is made from the mixed growth on the surface of the medium and organisms having the characteristic morphology of *Corynebacterium diphtheriae* are sought among the myriads of other organisms present. Considerable experience is necessary for this work (Figs. 250 and 251) for it is in such material that one often encounters diphtheroids.

There is another kind of diphtheria-like organism which may cause considerable confusion. It is harmless for guinea pigs but is morphologically and culturally *indistinguishable* from *C. diphtheriae*. This is spoken of as *avirulent C. diphtheriae*.

Virulence of a corynebacterium cannot, therefore, be inferred from its typical *Corynebacterium diphtheriae*-like morphology or cultural characters. The virulence or avirulence of the organism should be determined. This can be done by one of several methods. All involve the injection of the bacteria into guinea pigs or rabbits or 10- to 20-day-old chicks after isolation in pure culture. The use of mixed cultures often gives incorrect results.

Tellurite Media in the Isolation of Corynebacterium Diphtheriae. The isolation of corynebacteria from such highly contaminated material as cultures of the secretions of the throat or nose is greatly facilitated by the use of plates of blood agar containing *potassium tellurite*. Cystine, in small quantities, favors the growth of corynebacteria. Potassium tellurite, in concentrations of about 0.004 per cent, not only retards or completely inhibits the growth of many other bacteria but, due to little-known chemical reactions, the colonies of corynebacteria on tellurite media are characteristically black or dark gray with various concentric rings of a lighter color, depending on the species.[‡]

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[*] *Löffler's medium:* To 3 parts of serum add 1 part of 1 per cent dextrose broth. Tube and sterilize 1 hour in a slanting position without releasing steam or air pressure.

[†] *Pai's medium:* For the serum in the above, substitute whole hen's eggs, and for the broth substitute distilled water. Mix well and sterilize in the same manner.

[‡] *Cystine-tellurite medium:* (*McGuigan and Frobisher*)[18]

To 1000 ml. of melted infusion agar cooled to 45° C. add

Sheep or rabbit blood (defibrinated or citrated) . .	50 ml.
Cystine	25–50 mg.
0.3 per cent solution potassium tellurite.	150 ml.

Mix well and pour into Petri dishes in amounts of 12–15 ml.

Since the cystine does not dissolve readily, the flask should be agitated frequently while pouring the plates.

The percentage of tellurite given has produced good results, but since not all samples of this salt are exactly alike, the strength of the solution might require some modification according to condition. Potassium tellurite solution may be sterilized by autoclaving or filtration. *Do not heat tellurite and cystine together* as the solution will turn black and be ruined. Other media giving comparable results are described in the literature cited.[19]

The colonies of *Corynebacterium diphtheriae* on tellurite-cystine medium are usually opaque, gun-metal in color, domed, shining and circular and about 1 mm. in diameter (Fig. 346). Sometimes they are smaller and flatter and partly translucent. A very distinctive variety, now known as the *minimus* type[9, 20] forms colonies about 0.5 mm. in diameter, or less, often barely visible to the naked eye. These are often flat and translucent. Their growth and virulence are greatly amplified by the addition of a surface tension reducent (Tween-80) to the cultures. The organisms are rich in lipids, like tubercle bacilli.[21]

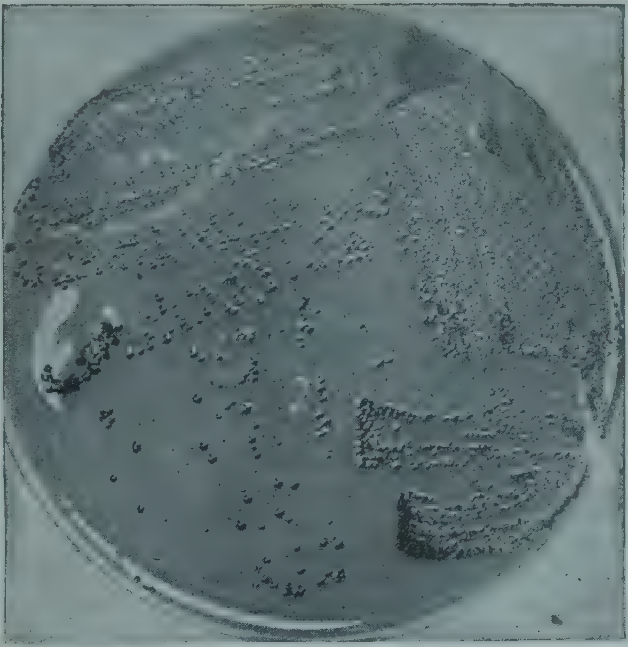

Fig. 346. Colonies of *Corynebacterium diphtheriae* on cystine-tellurite-blood agar. Plate was inoculated with a mixed culture on Löffler's medium, made directly from the throat of a patient and containing many different sorts of bacteria. This is a good example of selective bacteriostasis. (About ¾ natural size.)

While the various colonies may usually be recognized by one familiar with them, the morphology of *C. diphtheriae* growing on media containing tellurite is often very atypical so that, for final identification, as well as for virulence tests, the organisms must be transferred to Löffler's or Pai's medium. Once a pure culture has been obtained further studies of the organism are relatively simple.

The Virulence Test. For testing virulence the suspected organisms are cultivated in broth, or are grown on Löffler's medium and then suspended in 3 or 4 ml. of normal salt solution. Two tenths ml. of the culture is injected *into* (not under) the shaved skin of a guinea pig or rabbit. The resistance of guinea pigs or rabbits to ordinary throat bacteria and *avirulent* diphtheria bacilli when injected in this manner is such that the infection so produced, if any, is seldom severe, is localized and soon heals (Fig. 347).

If, however, *virulent* diphtheria bacilli are injected, a different and quite distinctive type of lesion is formed. After forty-eight hours a central area of ischemia (blanched) is visible, surrounded by a zone of erythema (redness). There is a little induration (swelling). The central area becomes necrotic (dead) and this is readily visible to the eye. This necrosis is quite widespread (1 to 3 cm.) and is not confined merely to the point at which the needle entered as is true of the occasional tiny abscess due to nontoxigenic bacteria. Eventually the necrosis extends and deepens and after seventy-two hours, if the animal is still alive, a definite ulcer is formed, covered with a crust.

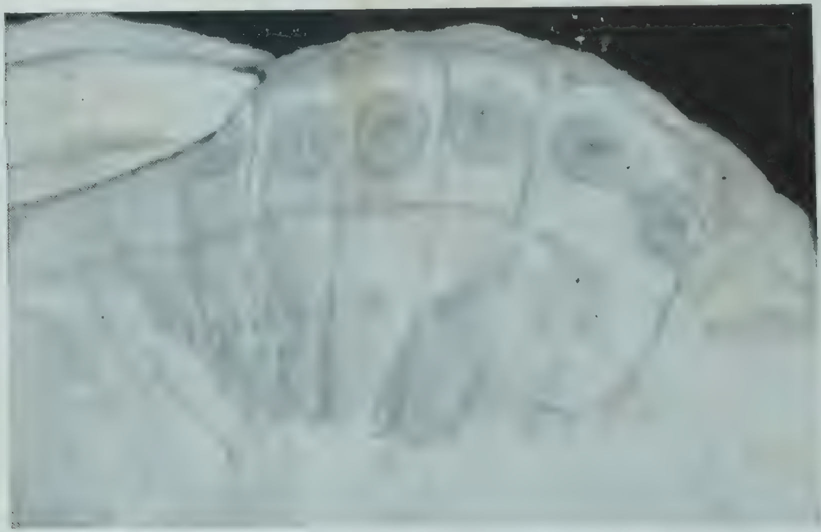

Fig. 347. Rabbit showing reactions of "virulent" cultures with controls. The dorsal skin of the rabbit was marked in squares with an "indelible" pencil. The test injections (0.2 ml. of culture) were made in the upper squares. Five hours later the rabbit received 1000 units of antitoxin intravenously and the same cultures were reinjected in the unused lower row of squares. This represented a specific control. Visible necrosis (large dark areas) developed in seventy-two hours at the sites of the first injections: very small, non-necrotic reactions appear at the sites of the control injections. (Photo courtesy Com. Dis. Center, U. S. Public Health Service, Atlanta, Ga.)

As a further check upon the truly diphtheritic nature of this reaction, *it must be shown that it will not occur in animals passively immunized with diphtheria antitoxin.*

Use of Chicks in Virulence Testing. As previously stated, 10- to 20-day-old chicks are highly susceptible to diphtheria and may conveniently be used to test virulence of diphtheria bacilli. The experiments are made with 3 ml. of forty-eight-hour broth culture injected subcutaneously or intraperitoneally. A control chick, previously protected with 50 units of antitoxin, receives the same injection of test culture.[22]

Chicks are much less costly than rabbits or guinea pigs, easier to handle, eat less, and require less space to house. They *must,* however, be kept warm and dry.

Types of Corynebacterium Diphtheriae. In 1931 attention was called to several different types of *C. diphtheriae*. Because of the rather constant presence of one type in severe (grave) cases of the disease it was thought to be the cause of severe cases and was called the *gravis* type (*B. diphtheriae gravis*). Another type, found at that time in less severe cases and in many healthy carriers, was thought to be less dangerous and was called the *mitis* type. A third type, found in moderately severe cases, and having some of the properties of both mitis and gravis types, was called the *intermedius* type.[23] In Baltimore, during an epidemic in 1944, a fourth type was found, characterized by very minute colonies and retarded fermentation of glucose. This was named the *minimus* type.[9, 20] The characters differentiating these types are shown in Table XX.

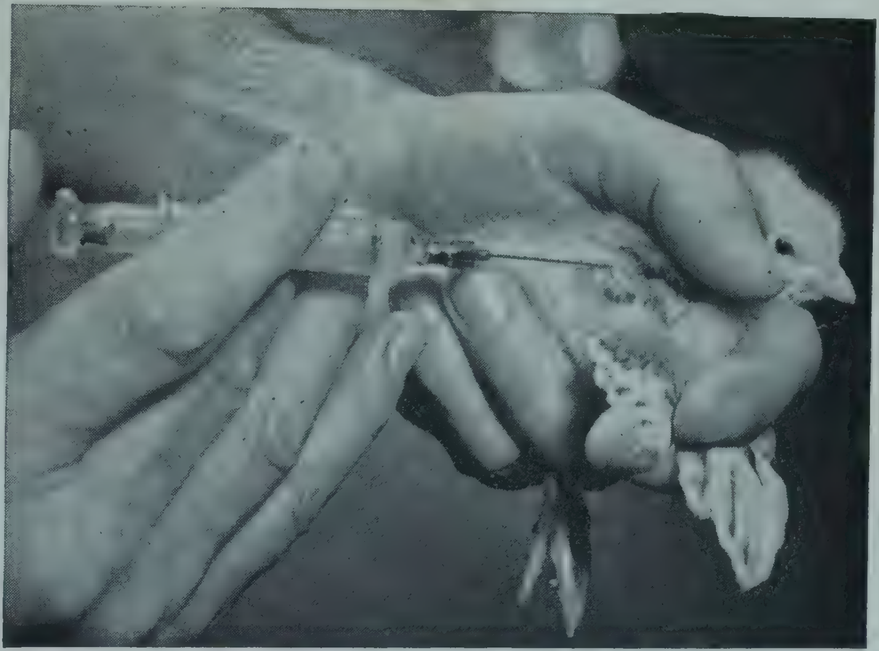

Fig. 348. The method of holding and injecting a chick in testing the virulence of diphtheria bacilli by the Frobisher procedure.

A large number of other types have been encountered, having various combinations of the listed characteristics. These characteristics

TABLE XX

CULTURAL PROPERTIES OF VARIOUS TYPES OF C. DIPHTHERIAE[*]

Type	Fermentation of Sucrose	Dextrose	Glycogen	Starch	Form and Diameter of Colony[†]	Hemolysis[‡]	Pellicle formation	Final pH of broth[‖]
Gravis	−	+	+	+	Low; conical; irregular margin and surface; (rough); 2–4 mm.	−	+	7.5–8.4
Mitis	−	+	−	−	Domed; smooth, regular; 1–2 mm.	+	−	6.5–7.4
Minimus	−	−	−	−	Surface and margin smooth; *very* minute; usually flat; 0.05 to 0.5 mm.	−	−	6.5–7.4
Various others not named[§]	−	−	+	+	Usually like mitis type	+ or −	+ or −	Variable
	+	+	−	−				Variable

[*] Any of these may be virulent or avirulent.

[†] On heated blood agar with tellurite.

[‡] Mix 0.5 ml. 48-hour broth culture with 0.5 ml. 2 per cent human erythrocyte suspension.

[‖] In absence of dextrose, after 5 days at 37° C.

[§] See reference no. 23.

are rather variable and are much affected by the kind of culture media used, history of the strain, and other factors. It has been proposed to give the various types numbers, but there is little agreement on the exact descriptions of any of them.[24] An important point is that several types (*minimus* and others) have fermentation reactions in glucose and sucrose broth which are like the reactions of some diphtheroids. This leaves the virulence test as the only reliable diagnostic test for the causative agent of diphtheria.[20, 25, 26]

As for the significance of these various types, it seems to be of interest only to the laboratory worker. There is no evidence at present of a constant connection between any cultural type (gravis, mitis, minimus, etc.) and either grave or mild diphtheria or the carrier state.[24, 27] Their prevalence may vary greatly from time to time and place to place.[28]

The toxin which they produce has been shown by Zinnemann and others[29] to be identical with ordinary diphtheria toxin. Many avirulent (harmless) gravis strains have been isolated from carriers in the United States.[28]

TUBERCULOSIS

Tubercles and Tuberculosis. When tubercle bacilli gain a foothold in a susceptible animal or person, the tissues where the bacilli localize immediately begin to react against the organisms in a very characteristic way. Numbers of tissue cells begin to grow around the bacilli in an attempt to incarcerate them or wall them off. This tiny, pearly gray mass of cells with tubercle bacilli at the center is called a *tubercle*. If the resistance of the host is low, the tissues are unable to arrest the bacilli and they continue to grow, killing the surrounding cells. Numbers of adjacent tubercles may coalesce. The dead tissue at the center of such masses of tubercles becomes cheesy and yellowish and is said to be *caseated*. If the tubercle is in a lung, the necrosis (death of tissue) may extend till it invades and breaks through the wall of a bronchus, and then the caseous material, which may contain millions of living tubercle bacilli, is discharged with the sputum by coughing (Fig. 30).

Often the caseous process breaks through the wall of an artery and then hemorrhage of the lung occurs, which may be fatal. In human beings these tuberculous processes tend to heal and to form life-long scars, whether the patient recovers or not.

Tuberculin Reaction. In a previous chapter it was pointed out that the presence of foreign proteins, including bacterial proteins, in the blood or tissues of the body often induces, in addition to antibodies demonstrable in the circulating blood, a hyper-reactive or hypersensitive condition known as an *allergic state* toward that specific protein. It was also stated that by injecting or scratching a small amount of the protein in question into the skin of a person allergic to that protein, a distinctive, local, inflammatory reaction will be elicited, showing the patient's allergic condition. These tests are highly specific for each protein.

This local reaction is very readily demonstrated in persons who have had tubercles in their tissues, whether healed or not. Sensitivity to the protein of the tubercle bacilli may be demonstrated in a large proportion of *adults* by introducing *into* (not under) the skin the tuberculoprotein in the form of dead tubercle bacilli, or the sterile filtrate of broth in which they have grown, or purified protein derivatives (P.P.D.) extracted from the bacilli. These tuberculo-proteins are called *tuberculin*. At the site of their introduction into the skin a red, itching spot appears in twenty-four to forty-eight hours. The reaction is called a *tuberculin reaction*.[30, 31]

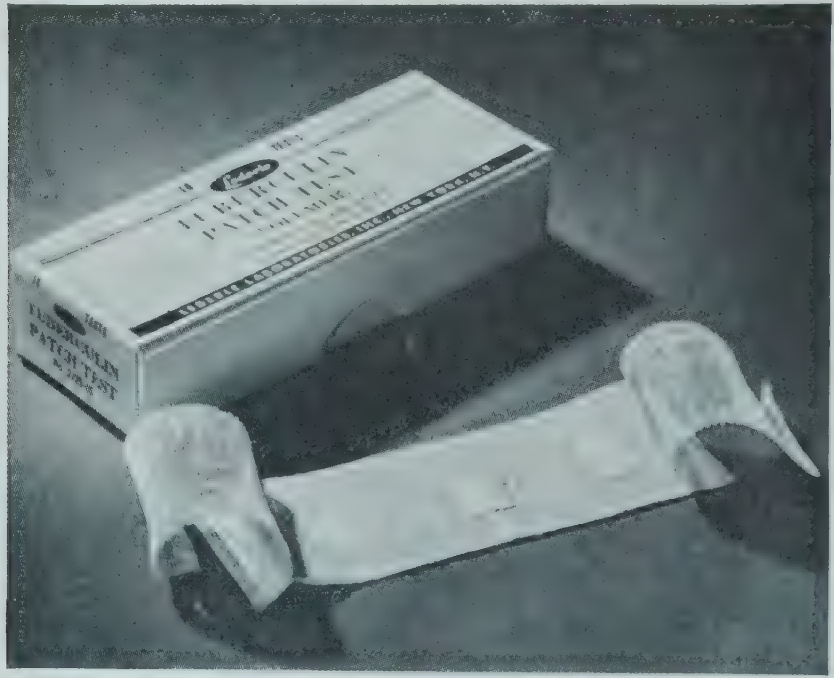

Fig. 349. Tuberculin patch test (Vollmer).

Tuberculin Reaction and Resistance. When a tuberculous infection progresses to the time when hypersensitiveness appears, a certain degree of resistance to later infection develops. Tuberculin tests become positive at the same time. Evidently the hypersensitive tissues are able to react locally to bind and arrest the further progress of new infection.[32, 33, 34, 34a]

Skin Tests Related to Tuberculin Test. Skin tests with two other antigens are of importance in connection with studies of the distribution and control of tuberculosis.[35] In certain areas of this country, notably Tennessee, Kansas, Iowa and other states in the Mississippi basin, many persons were found who had x-ray pictures indicating tuberculous processes in the chest but whose tuberculin reactions were negative. It was later found that they gave positive reactions to *histoplasmin,* a preparation analogous to tuberculin, but made from *Histoplasma capsulatum* (see page 407). It is now thought that *Histoplasma* infections are common and may simulate tuberculous lesions as seen in x-ray pictures. The histoplasmin test is, therefore, of great value as an accessory in studies of tuberculosis.[35a]

A similar situation exists with respect to *Coccidioides immitis.* In regions where this organism abounds chest x-rays may suggest tuberculous disease in persons whose tuberculin test is negative yet who give a positive reaction to coccidioidin (see page 405).

The student should carefully distinguish between (*a*) the Schick and Dick tests, which test for *susceptibility* to *toxin,* and are used generally *before* infection sets in, and (*b*) the tuberculin, histoplasmin and coccidioidin tests, which are used to detect *allergy* to an *infection already present* or healed. Skin tests for allergy, similar in principle to the tuberculin test, are used in various other microbial infections; *e.g.,* tularemia and brucellosis, but are of undetermined significance.

Immunization Against Tuberculosis. Many attempts have been made to produce an effective bacterial vaccine to prevent tuberculosis.

The most widely used and favorably reported immunizing agent is B.C.G., a variant of a bovine strain of tubercle bacilli of low virulence (see page 475). Because of continued favorable results in this country, Europe and South America, the United States Public Health Service Tuberculosis Control Division, in conference with many experts, decided in 1946 to undertake more extensive studies with a view to improving methods and obtaining complete data.[36, 37] The safety of B.C.G. is generally acknowledged. It is probably of great value. Suspensions of the live attenuated organisms are fed or injected subcutaneously or applied in superficial punctures. Excellent results have been reported in a number of studies.[38]

In 1937 a third mammalian type of *Mycobacterium tuberculosis* was described in tuberculosis of voles (see page 471). Tests have been made of its value in the form of a living vaccine as an immunizing agent in human beings against human tuberculosis. It appears to be as safe and effective as B.C.G. Extensive trials have not been made in the United States. All of these vaccines produce a *positive tuberculin reaction;* a desirable result.[39]

LEPROSY

Although not common in the United States or in most of the so-called civilized countries, this disease is of great interest from several standpoints: (*a*) historically, (*b*) because of the mystery surrounding its mode of transmission, and (*c*) doubt concerning its true etiology.

The disease is, of course, well known to students of the Bible and medieval history. The fear and horror of lepers has been a human tradition since antiquity. In ancient times lepers were excluded from all public contacts and left to die of exposure and starvation by many peoples. They still are excluded and shunned, but provisions for their comfort and well-being are usually adequate. One of Conan Doyle's most intriguing Sherlock Holmes episodes depicts the tragic segregation of a noble young man because of a suspicion of leprosy. Holmes, with the aid of Watson, dispels the ugly cloud by his usual astute, deductive reasoning, and the victim is found to have nothing but a harmless skin condition called ichthyosis. The horror of leprosy arises from the disfigurement of the body and the destruction of tissue, with scar formation, which accompanies it (Fig. 350).

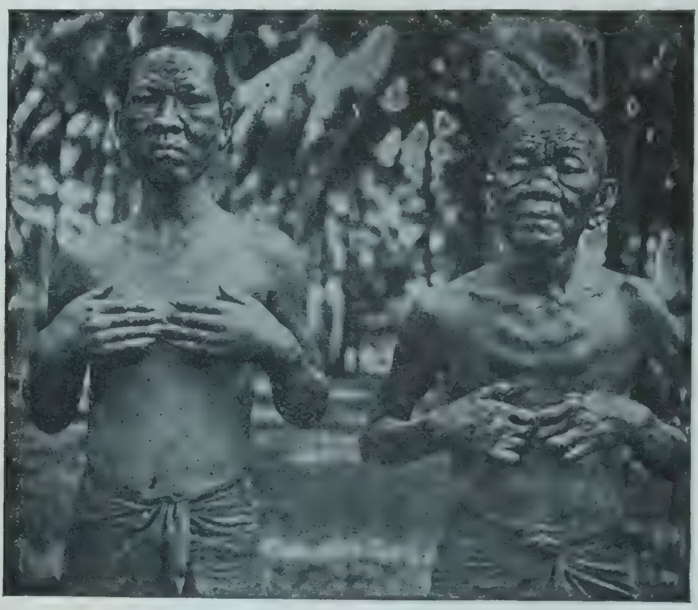

Fig. 350. Untreated case of nodular leprosy, showing advanced lesions of face and hands (Kuala Lumpur). (MacCallum.)

Etiology. In 1874 Hansen described an acid-fast bacillus, morphologically indistinguishable from *M. tuberculosis*, in lesions of lepers. It is called *Mycobacterium leprae*. It is always present in leprous tissue, and never occurs in normal tissue. For many years this has been the only one of Koch's postulates to be demonstrated in connection with the etiology of leprosy. Recently several reports of successful inoculation of human beings with leprous material have appeared.[38, 39] It still is not certain that the *only* infectious agent transferred in these occurrences was the bacillus of Hansen. Moreover, all of these people were in *constant association* with lepers. Inoculation of leprous tissue into chimpanzees, etc., has never produced an authentic case of leprosy. An organism which will cause leprosy has never been cultivated, although many claim to have done so. There are several strains of so-called *M. leprae* which may be obtained on ordinary culture media. They have all the properties of saprophytes of the environment and no one has produced leprosy with them. They appear to be harmless. Their true relation to the disease is unknown.

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Chapter 44

THE GENUS NEISSERIA

The family Neisseriaceae includes two genera, *Neisseria* and *Veillonella*. The latter are small, gram-negative, anaerobic cocci which occur chiefly in the normal mouth. The group has been relatively little studied and will not be discussed in detail.

Genus Neisseria. The genus derives its name from a German bacteriologist, Neisser, who, in 1879, discovered and studied one of the two most important species, *N. gonorrhoeae;* the cause of gonorrhea and commonly known as the gonococcus.

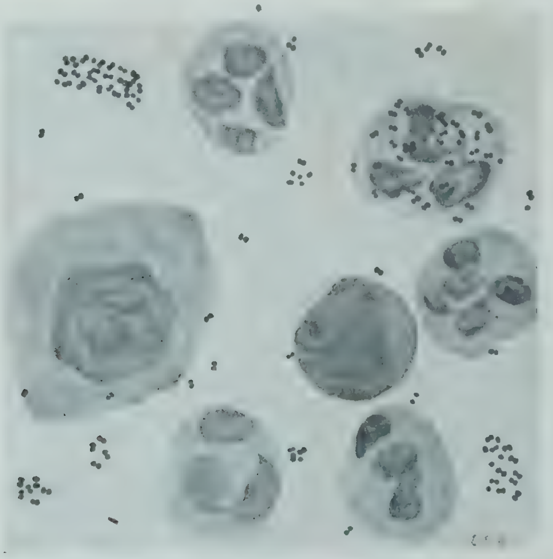

Fig. 351. Gonococcus from urethral pus, stained with methylene-blue. (Ford: Textbook of Bacteriology, W. B. Saunders Co.)

The *Neisseria* are rather small, gram-negative diplococci, each cell characteristically flattened where it is in contact with its mate, each cell having somewhat the shape of a coffee bean (Fig. 351). They are aerobic and grow best at 35° to 37° C. All but one, *N. gonorrhoeae,* are inhabitants of the normal human respiratory tract and are not found on other animals or soil, water, etc.

Of the several species of *Neisseria*, three commonly cause disease. *N. gonorrhoeae* causes gonorrhea, while *N. meningitidis* (also commonly known as the meningococcus) causes epidemic meningitis.

N. flavescens is also encountered occasionally as the cause of this disease. *N. catarrhalis,* as its name implies, was originally thought to be involved in catarrhal conditions of the upper respiratory tract, but is now regarded as of little or no pathogenic significance except in certain special circumstances to be described.

The gonococcus and meningococcus have long been thought to be highly selective and fastidious in the matter of artificial culture conditions,[1, 2, 3] requiring blood or serum and meat infusions and peptone for growth.

It now seems probable that the role of blood or serum in such media is not nutritive but to nullify the inhibitory effect of constituents of peptone and other ingredients. The organisms can be cultivated on a very simple dilute solution containing a mineral supplement ($NaCl$, Na_2HPO_4, KH_2PO_4, NH_4Cl, $FeSO_4$, $MnSO_4$, $MgSO_4$ and $CaCl_2$) with sodium glutamate, thiosulfate and glucose.[4] It may be, however, that for initial isolation from pathological material the more complex media are preferable as mentioned later on. Several such media are recommended.[5]

In moist, warm material, as pus, urine, serum or cultures in incubators, these organisms autolyze and die rather rapidly. They do not survive for long periods outside the body as do typhoid, tubercle and diphtheria bacilli. However, gonococci may survive some hours in certain protective solutions designed to prevent overgrowth of contaminating organisms which kill gonococci, especially if kept cold (4° C.) to prevent rapid action of autolytic enzymes. The perfection and use of such solutions is an important problem of laboratories engaged in diagnostic work when the specimens must be shipped from a distance.[6] Drying is very deleterious to gonococci. Meningococci are very sensitive to low temperatures but less so to drying; they can live for days dried on coverslips. Sunlight and diffuse daylight are very destructive to them.[7]

These characteristics serve to differentiate the meningococci and gonococci from all other species of *Neisseria.* These others are usually thought of as minor pathogens. They grow well on blood-free media at temperatures as low as 25° C. and are moderately resistant to drying and light. These species, common among which are *Neisseria sicca* and *N. flava,* while parasites in a broad sense, being restricted to a life on a mammalian host, are usually quite harmless. As shown by Sophian and others, however, under certain conditions some of them may cause meningitis,[8] and some can cause vaginal infections in little girls which are sometimes confused wtih gonorrhea with tragic results. *N. flava* and *N. sicca,* as well as *N. meningitidis,* have been thus incriminated. Therefore, no diagnosis of gonorrhea can be said to be complete and accurate without a full bacteriological study of the organism involved. This is especially true of institutional outbreaks of vulvovaginitis in little girls as shown by Carpenter and Nelson and others, may sometimes consist entirely of infections due to *N. catarrhalis* or *N. sicca*.[9, 10, 11]

The *Neisseria* are all very similar, and may represent merely varieties of one species. Microscopically they are indistinguishable. The colored species (*flava, perflava, subflava,* etc.), produce colonies with greenish or yellow pigment, while the colonies of *N. sicca* are of a dry, crumbly consistency.

Neisseria flavescens[12] is a serologically homogeneous and distinct species and is easily distinguished from *N. gonorrhoeae* and *N. meningitidis* by its ready growth, by agglutination tests, its yellow pigment, and fermentation tests. In many respects it resembles *N. catarrhalis.*

TABLE XXI

DIFFERENTIATION OF NEISSERIA

Species	Glucose	Sucrose	Maltose	Growth at 25° C.	Growth on plain extract agar
Neisseria gonorrhoeae	+	−	−	−	−
Neisseria meningitidis	+	−	+	−	−
Neisseria catarrhalis	−	−	−	+	+
Neisseria flavescens	−	−	−	+	+
Neisseria sicca	+	+	+	+	+
Neisseria flava	+	−	+	+	+

N. meningitidis and *N. gonorrhoeae* are closely related to each other, being distinguished with difficulty even by serological means and fermentation tests (see Table XXI). Indeed, *N. gonorrhoeae* has been found to cause meningitis while *N. meningitidis* has been isolated from conditions clinically indistinguishable from gonorrhea.

Cultivation of N. gonorrhoeae and *N. meningitidis* from pathological material requires special media and great care.[13] It is desirable to use moist, freshly prepared, "chocolate" agar (add 5 per cent blood to melted infusion agar at 85° C.—blood turns chocolate brown).* The pus from gonorrheal infections, or sediment from spinal fluid, is streaked on the surface of the chocolate agar in plates, and the plates are then incubated in an atmosphere containing about 10 per cent carbon dioxide (Fig. 352).

Oxidase Reaction. The *Neisseria* possess a distinctive biological property which aids in their detection on the surface of blood-agar plates containing many different kinds of colonies. They produce an enzyme (oxidase) which causes a 1 per cent solution of dimethyl-paraphenylene-diamine to turn, successively, pink, rose, magenta, and finally black (Fig. 353). The solution is flooded (1 ml.) over a portion of the surface of a 24-hour-old chocolate agar plate prepared as suggested above, and previously inoculated with, for instance, a swab from the cervix uteri in an attempt to isolate gonococci. Colonies, which are at first colorless, translucent, flat and about 0.5 to 2 mm. in diameter, are noted and, in the unflooded portion, similar colonies are fished as soon as the first rose tint is observed.* These will grow in subcultures. If fishing is delayed too long, the cells are killed by the reagent. For subcultures infusion agar slants with heated blood are good.† For fermentation tests infusion agar containing 10 per cent serum, 1 per cent of the desired carbohydrate, and an indicator are used. The *Neisseria* seem to prefer solid media.

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* Suitable media are available commercially.

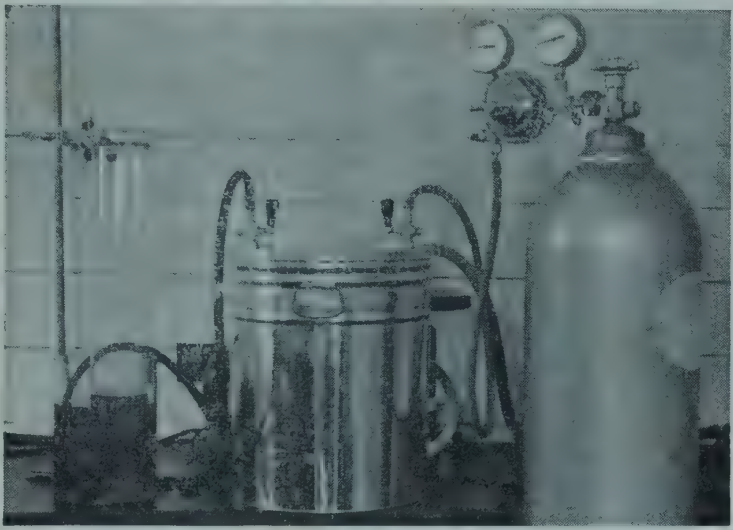

Fig. 352. Simple method of providing an atmosphere with approximately 10 per cent CO_2 for cultivation of various bacteria, including *Neisseria* and *Brucella.* The CO_2 is admitted to the culture vessel (center) containing Petri dishes, tubes, etc., from the tank of CO_2 (right). The displaced air is measured by water displacement in the graduated 2-liter cylinder (left). The volume of the culture vessel, containing cultures, is measured beforehand. Photo courtesy Federal Security Agency, P.H.S., C.D.C., Atlanta, Ga.

Plates inoculated with swabs from the nasopharynx (the habitat of the meningococcus) often contain *N. sicca, N. catarrhalis,* and related species, all of which give the "oxidase reaction" and thus make the test inapplicable to cultures from the respiratory tract if differentiation of the meningococcus is desired. Cultures from the genitalia rarely contain more than one type of *Neisseria*. If more than one type are found, all may be of diagnostic significance. In meningitis, cultures from the sediment of centrifuged spinal fluid are made in the same manner. Once isolated, a *Neisseria* may be identified by the characters shown in Table XXI.

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* If flooded colonies are fished they are apt to be found contaminated by organisms washed to them by the oxidase reagent.

† Good subculture media are available commercially.

The meningococci are further identified by, and separable into, four main serological types on the basis of agglutination reactions. These are types I, II, II_a and IV. A "Quellung" reaction for "typing" is available, analogous to that used in typing pneumococci, since freshly isolated strains of meningococci possess capsules. The method has been used effectively by Cohen and others.[14] It has been found by Phair[15] and others[16] that the serum of chickens immunized with meningococci of various types gives highly specific agglutination and Quellung tests. These methods have been used effectively in epidemiological studies.[17] The value and importance of continued typing even though chemotherapy has greatly lowered the mortality rates in meningococcal meningitis, has been clearly stated.[18]

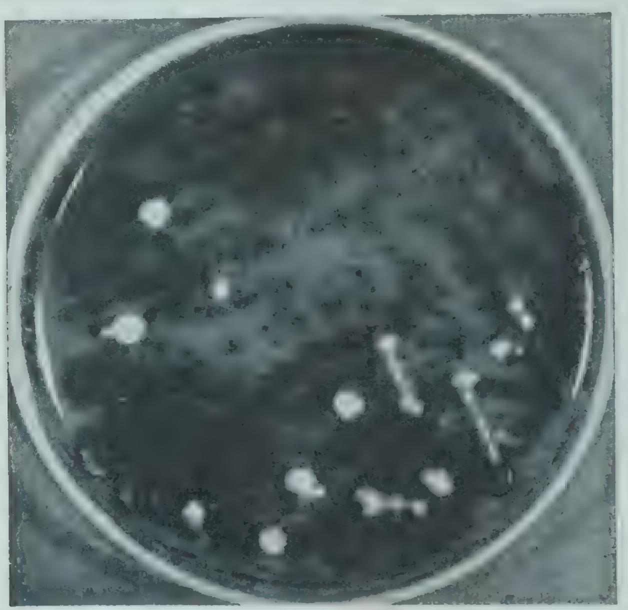

Fig. 353. The oxidase reaction of *Neisseria*. Most of the colonies are oxidase-positive, having darkened the reagent applied to the plate. (Steinberg and Mollov, J. Lab. and Clin. Med., vol. 27, C. V. Mosby Co.)

PATHOGENIC ACTION OF THE NEISSERIA

Gonorrhea. This is one of several diseases commonly spoken of as "venereal diseases," deriving this appellation from the name of Venus, goddess of love. The inappropriateness of this term will become obvious in the discussion of the infections.

Gonorrhea is an inflammatory disease due to infection by *N. gonorrhoeae,* of the mucous surfaces of the reproductive organs of both men and women. Much pus forms, and appears as a white discharge (leukorrhea) from the genitalia. It is an alarmingly prevalent disease, hundreds of thousands of cases being under medical care in the United States in 1949. There are undoubtedly many other, unreported and untreated cases.

Infection with *N. gonorrhoeae* occurs mainly through sexual intercourse, transmission by clothing or other fomites being so rare as to be negligible. Gonorrhea is seldom fatal but is sometimes difficult to cure. Patients often believe themselves cured only to find, later, that the disease has reappeared in a chronic form. The sulfonamide drugs promised for a time to eliminate gonorrhea, but drug-fast strains rapidly developed and indiscriminate use of the drugs has robbed such therapy of much of its effectiveness because of adaptation to the drugs by the gonococci.[19] Special tests are used to detect drug-fast strains.[20] Penicillin is used in treating infections with such strains,[21] and indeed penicillin now offers the best hope for cure in all cases.[22] It can be readily understood that careless, ignorant or malicious people can spread gonorrhea widely. Prostitution is one of the chief means by which the disease is propagated. It has been shown by Eagle, et al., that treatment with penicillin *very close* to the time of exposure will prevent many cases from developing.[22a]

Gonorrhea, untreated, often results in sterility. The intense inflammation caused by the endotoxins of the organism destroys the lining tissues of the genito-urinary tract, the tissue being replaced with scars. Such scars obstruct the *fallopian tubes* of the female and the *vas deferens* of the male (tubes through which the reproductive cells pass). Such scarred obstructions are called *strictures*. Stricture of the urethra in the male interferes with urination and may require surgical intervention. Gonococci sometimes invade the body, localizing in the joints and the heart valves. In the former case, a very painful and stubborn type of arthritis (rheumatism, or inflammation of the joints) results while in the latter case a very damaging disease of the heart occurs, with permanent injury and sometimes death.

Gonorrheal Ophthalmia. An inflammation of the eye results when gonococci are rubbed into the eye. Loss of sight usually results unless treatment is prompt. A gonorrheal mother may infect her child at birth. Due to this fact most cities, states and countries require that physicians, nurses or midwives attending births, *regardless of any circumstances,* instill into the eyes of the infant a few drops of weak silver nitrate or other appropriate disinfectant solution. This rapidly destroys gonococci. Approved disinfectants are obtainable at any health department or drug store, ready for use. In Baltimore the use of penicillin solution has been adopted.[23]

Laboratory Diagnosis of Gonorrhea. The diagnosis of *acute* gonorrheal infection in the adult male is usually based on microscopic examination of the pus. The pus is stained by Gram's method and the gonococci appear as gram-negative, coffee-bean-shaped diplococci *within* the leukocytes (Fig. 351). Capsules can be demonstrated by staining with Wright's stain (Fig. 354).[24] Such organisms in the *adult male* in *acute urethritis* are rarely any but gonococci. In the female genito-urinary tract many other organisms are present and, as noted above, other *Neisseria* may cause infection in little girls. Isolation of the organism is therefore of importance although smears of pus from cervical glands, etc., may give a valuable clue, especially in fresh cases in adult women and when the patient admits exposure to infection. In any doubtful or chronic case, or little girls, plate cultures are essential.

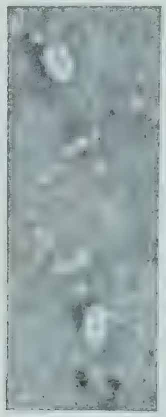

Fig. 354. Capsule of *N. gonorrhoeae* stained with Wright's stain (× 1000). (Bernstein, Proc. Soc. Exp. Biol. and Med., vol. 46.)

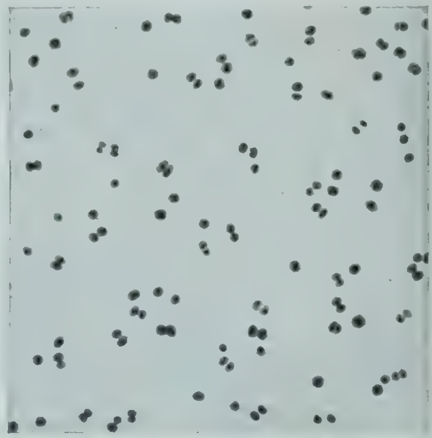

Fig. 355. Meningococcus from culture on blood agar twenty-two hours old, stained with Gram and carbol-fuchsin. (Ford.)

One difficulty has already been mentioned; the necessity, especially in health department practice, of sending material for culture through the mail, etc. The mailing of already inoculated chocolate agar slants may be of help in such work.[25]

Another difficulty in cultural diagnosis in women is that in the latter part of the luteal phase of the menstrual cycle, when the cervical mucus is acid and progesterone activity is high, negative results are frequently obtained while in the estrogenic phases cultures will often be positive.[26] Unless attention is given to this very important detail, cultures are likely to be negative even in the known presence of infection.

Meningitis. The term meningitis is a pathological one and means, simply, inflammation of the membranes (meninges) covering the brain and spinal cord. It may be due to mechanical irritations, viruses, or any of several kinds of bacteria which may localize in the meninges. Among the bacteria often found are hemolytic streptococci, tubercle bacilli, pneumococci, influenza bacilli (*Hemophilus influenzae*) and occasionally a variety of other organisms, such as *Salmonella typhi, Escherichia coli,* various "saprophytes," and *Pseudomonas aeruginosa.* These are all more or less accidental infections, following usually upon extensive invasion of the blood stream after severe cases of infection of other parts of the body by the organisms involved. The meningococcus is the only common cause of *epidemics* of meningitis.

Epidemic Meningitis. Meningitis is frequently due to the meningococcus. Carriers of meningococci are common, but meningitis is not.[27] There is evidence that the meningococcus very often causes conditions like rhinitis, "catarrh" or purulent "colds" which heal and attract no particular attention because the etiological agent is unsuspected. The carrier state may quickly be cured with sulfonamide drugs.[15, 28]

Laboratory Diagnosis of Meningitis. Diagnosis of meningococcus meningitis is made by examining gram-stained smears of the sediment in fluid from the spinal canal. The presence of the gram-negative, bean-shaped diplococci *within* the leukocytes suggests the diagnosis (Fig. 351). However, as stated before, gonococci and *N. flavescens* can also cause the disease and microscopic examination alone is not sufficient to establish the identity of the organism. The organisms may be cultivated on the same sort of media as are used for gonococci (Fig. 355).

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Chapter 45

THE "LITTLE BACTERIA"

The family Parvobacteriaceae ("little bacteria") was established to provide a convenient grouping for a number of true bacteria which are relatively small (0.3 μ by 2 μ), gram-negative, non-sporeforming, parasitic, aerobic or facultative rods. Some are anaerobic or facultative; some are motile, others non-motile. They seldom are found thriving in environmental habitats; *i.e.,* outside the human or animal body, except species (such as *Pasteurella pestis*) capable of existence in an insect host. As a group, they represent (especially the genera *Pasteurella* and *Hemophilus*) the highly parasitic type of bacteria requiring body proteins (blood serum or tissue) or complex organic media for their optimum growth. Further, many are rather sensitive to light, cooling, drying, etc., and lack the ruggedness necessary to growth and survival in the outer world which characterizes such organisms as the *Aerobacter, Serratia* and *Pseudomonas.* Generally speaking, they have little fermentative or proteolytic power, and do not cause notable changes in carbohydrate or protein media.

The family comprises some of the most dangerous pathogens such as *Pasteurella pestis* (the "plague bacillus"), *Past. tularensis* (the organism of "rabbit fever" or tularemia), *Malleomyces mallei* (the cause of glanders in horses and man), three species of *Brucella* (causing undulant fever in man and infectious abortion, etc., in animals) and *Hemophilus pertussis* (the cause of whooping cough).

There is a very extensive bacteriological literature concerning all of these organisms and an equally imposing medical literature about the diseases they cause. In the present discussion, only such of them and their properties will be described as serve to illustrate special methods of cultivation or fundamental bacteriological or biological principles.

GENUS PASTEURELLA

Pasteurella Multocida. *Species Causing Hemorrhagic Septicemia.* The genus is named for Pasteur, who founded the science of immunology on his studies of vaccination again the cause of fowl "cholera," which was for long known as *Pasteurella avicida.* This species is now regarded as a variant of the type species, *Past. multocida.* Other variants of *P. multocida* have previously been named for various animals in which they have been found; for example, *P. cuniculicida* (rabbits); *P. bovicida* (cattle); *P. suilla* (swine); *P. muricida* (rats); etc. These vary somewhat in fermentation reactions but there is no good reason for regarding them as species distinct from *P. multocida.* They are all pathogenic for birds and animals, but not for man.

The organisms invade the blood stream and may be cultivated on infusion media from all the organs. There are many small hemorrhages on various internal mucous surfaces, on the skin, and in the internal organs, hence the name "hemorrhagic septicemia." There is much exudation of fluid from nose, mouth, eyes, etc. This fluid is highly infectious and transmits the disease which is rapidly fatal as a rule.

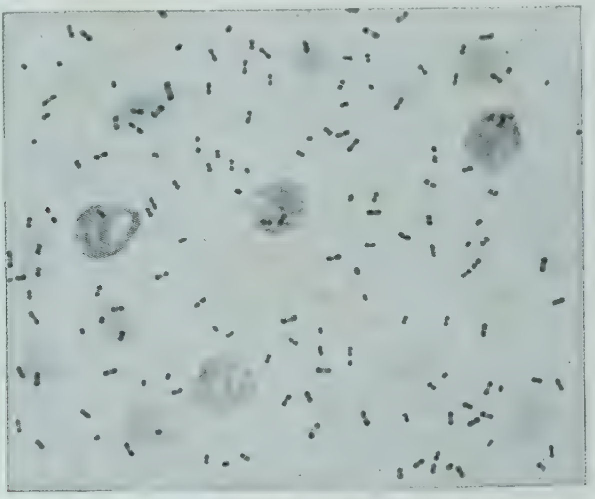

Fig. 356. *Pasteurella multocida* from heart blood of infected rabbit, stained with methylene blue (× 900). (Ford.)

Culturally, the organisms are not refractory, growing well on infusion agar or serum agar and in ordinary infusion broth. They are not very active biochemically, fermenting few carbohydrates and not hydrolyzing gelatin. They are aerobic.

The morphology of the organisms is rather characteristic. In pathological material they are short, oval rods, about 0.5 micron by 3 microns, which tend to stain most heavily at the tips. This gives them a distinctive appearance (bipolar staining, Fig. 356). In culture media the bipolar appearance is less definite. They are not motile. They succumb readily to heat and disinfectants but resist drying to some extent and therefore are often the cause of severe and costly epidemics among laboratory and farm animals. These epidemics can be controlled only by rigid isolation, disinfection, and killing sick animals followed by incineration or burial.

Past. multocida has been found in the mouths of wild rats. It is curious that the rats are completely resistant to the organisms, while other animals were very susceptible.[1] Presumably the bites of rats may be very dangerous to animals for this reason.

Pasteurella Pestis. From the standpoint of mankind, the most important member of the genus is *Past. pestis,* the cause of bubonic and pneumonic plague in man.[2] Morphologically and culturally, *Past. pestis* resembles the other members of the genus closely but is slower and less vigorous in its growth and less active in fermentation.

Bubonic Plague. Bubonic plague, due to *Pasteurella pestis,* is an outstanding example of a bacterial disease transmitted by the bite of an insect. It is conveyed to human beings by the bite of infected fleas (Fig. 357), most important of which, in this respect, are *Xenopsylla cheopis* and *Ceratophyllus fasciatus,* the rat fleas. The insects usually derive the plague bacilli from the blood of the rats *Mus norvegicus* (sewer rat) and *Mus rattus* (house rat) which may be infected and which often show lesions similar to those in man (see page 771). Rats maintain plague as an epizootic among themselves for long periods and act, therefore, as an *animal reservoir* of plague bacilli. When rats become excessively prevalent in any locality, human plague is apt to occur because the opportunity for rat fleas to bite human beings greatly increases. Populations living near dumps or in dirty unsanitary conditions suffer most. Conditions following the devastation of war, with breakdown of disease-control systems, are ideal for the development

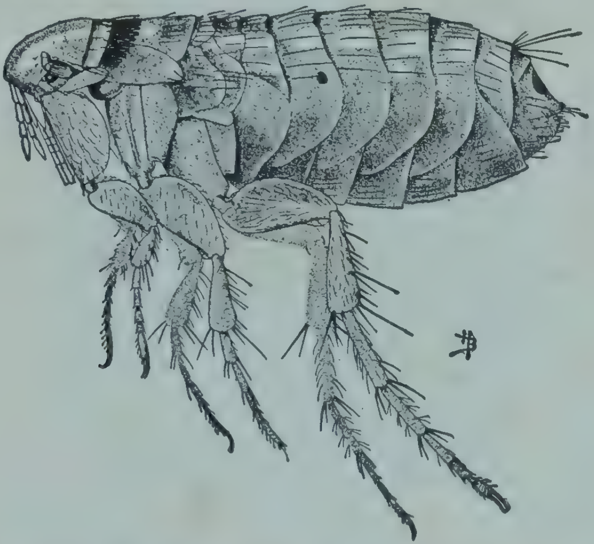

Fig. 357. The European rat flea (*Ceratophyllus fasciatus*): Adult female, greatly enlarged. (Bishopp: Bull. 248, U. S. Dept. of Agriculture.)

of rats and, therefore, rat-borne diseases.[3] The pages of history are filled with disasters to armies and civil populations attacked by plague. The rats often die in great numbers from the disease and the fleas tend to leave the cooling bodies, jumping on to the first warm animal which passes. Dead or dying rats, therefore, are potentially dangerous. Further, plague bacilli can survive for months in the bodies of dead animals so that these should not be handled carelessly if plague is suspected.

Since 1900, plague has been found in the United States in rodents other than rats, especially ground squirrels or "prairie dogs." Many human cases have been traced to wild rodents. The disease in woodland or wild-living creatures is spoken of as sylvatic plague.[4, 5] The control of wild rodents is a field problem of great importance.

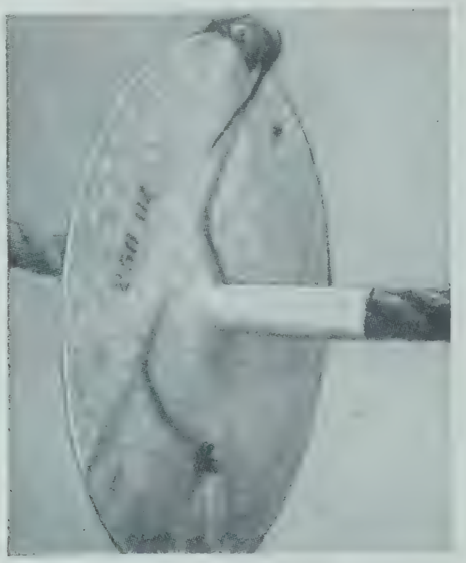

Fig. 358. Rat climbing over guard placed on ship's mooring line. (Denney, O. E., U. S. P. H. S., Weekly Reports.)

Plague is traditionally a disease of travel routes and, in rats, accompanies caravans, armies, and ships to foreign places. The United States Public Health Service frequently institutes "rat surveys" of seaports in the United States with the object (*a*) of determining the prevalence of rats, and (*b*) of determining the percentage of rats infected. The work is hazardous and should not be indulged in by amateur bacteriologists. The rats are trapped and inspected for infected fleas or for lesions of the disease,* or both. When either factor reaches too high a level, anti-rat campaigns are instituted. Traps are set, garbage dumps are cleaned, buildings are "rat-proofed" and numerous other precautions are taken. The ropes used to moor ships from foreign countries to wharves are always required to be guarded by means of large metal disks for the purpose of preventing rats from coming ashore. The rats often circumvent this, however, by dropping into the water and swimming ashore, or by climbing over the guard (Fig. 358).

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* Principally enlarged lymph glands (buboes). Smears and cultures may show the bacilli.

Plague in Man. The disease in human beings is characterized by the usual symptoms of an infection and often by a pustule developing at the site of the infecting bite. The pus teems with plague bacilli (Fig. 359). The bacteria travel along the blood and lymph vessels and are taken up by the nearest lymph glands situated in the groin, arm pits, neck, etc., where secondary foci of infection occur, the glands becoming greatly enlarged. They are then called *buboes* (hence *bubonic*) and are filled with a "puree" or thick pus teeming with bacilli. Material taken from the buboes, or from the blood, may be smeared, cultured, or injected into guinea pigs in which the organisms cause a bubonic and hemorrhagic disease similar to that seen in rats and man.[9]

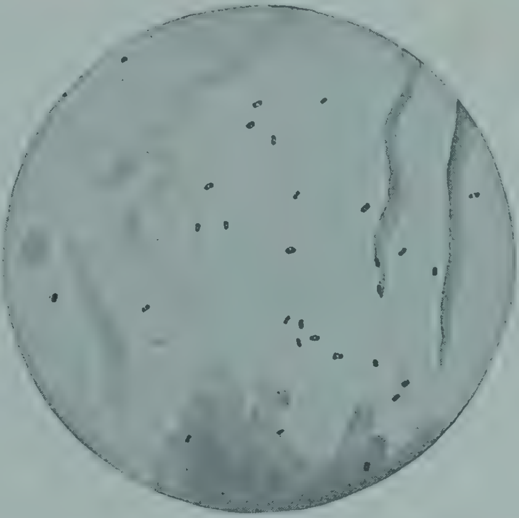

Fig. 359. *Pasteurella pestis* in pus from the site of a flea bite on the ankle of the sick woman mentioned in Fig. 360. Note the bipolar staining of the oval rods.

Today, human plague is rare in North America but is found in the Orient, some cities of South America (Fig. 360) and Central Europe. The pneumonic form of plague is particularly fatal, and is transmitted by coughing and sneezing. It develops when the organisms localize in the lung tissues from other sources in the body, and are then excreted to the exterior in the pus and mucus of the sputum. An outbreak of the pulmonary form of plague in Mukden was carefully described by Lieh, et al., with good descriptions of the disease and steps taken to control it.[9a]

Prevention of plague obviously depends chiefly upon suppression of rats and this in turn is based on good municipal sanitation and avoidance of conditions favorable to rat multiplication. Vaccines of killed bacilli are said to be effective but only to a limited extent. They reduce *mortality*, but not *morbidity*. Streptomycin appears to be very effective in the treatment of plague.[10] So also does sulfadiazine.[9a]

Pasteurella Tularensis. This member of the genus is much like *Past. pestis* in most respects. It is more exacting in its nutritional requirements than the latter. Growth is much enhanced by the amino acid cystine or (what amounts to the same thing) by media containing compounds with the —SH (sulfhydryl) group.

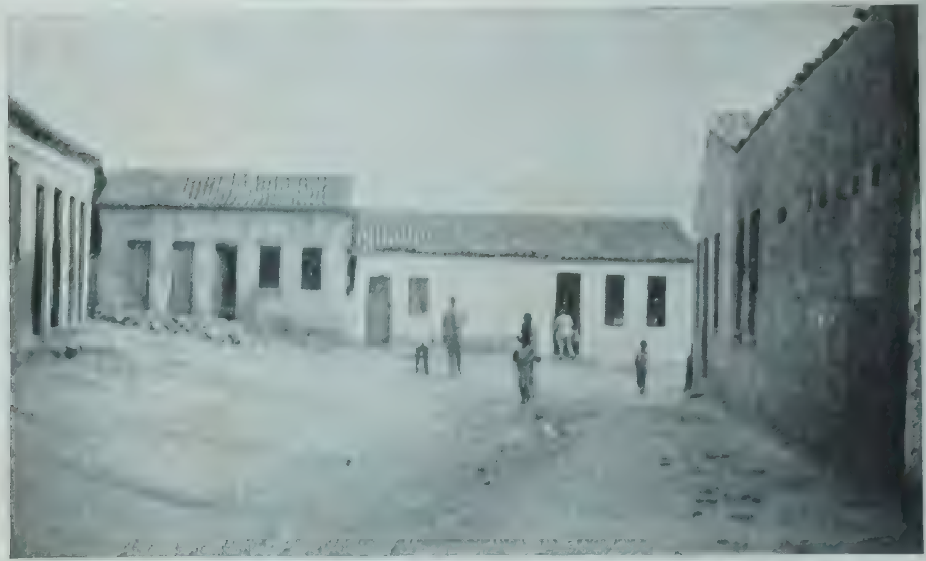

Fig. 360. A plague-infested town in Brazil. A woman lay dying in the house at the extreme right; two girls were recovering with draining buboes, in the house at the extreme left, while a boy and girl (seen in the windows) had fully recovered and had scars in the groin, in the house where the man is entering. Two dead rats were found in the gulley in the center foreground. The bacilli seen in Fig. 359 were obtained from the dying woman.

On ordinary blood agar it rarely grows on first isolation, but if a small amount of cystine be added, growth will usually occur. The organism grows well in embryonated eggs.[11] Several simplified liquid media have also been used successfully.[12]

Past. tularensis is found in much the same ecological relationship to rodents (rabbits, gophers, mice), biting insects (wood, dog, and rabbit ticks, rabbit lice, deer-flies, horse flies) and man as is *Past. pestis*.[13, 14] It causes a disease called tularemia (Fig. 361).

Tularemia ("Rabbit Fever"). This disease is common in many American rodents and other animals, in which it causes a plaguelike disease, with glandular enlargements, swelling of the spleen and the appearance of whitish nodules in the liver, spleen and elsewhere. These somewhat resemble tubercles in gross appearance. The bacilli invade the blood stream from these foci just as *Past. pestis* does in plague, and thus biting insects, especially various ticks, flies and rabbit lice, may transmit the organisms from one animal to another and to man. The disease in man is analogous to that in animals, but is much less likely to end fatally. One common means of transmission

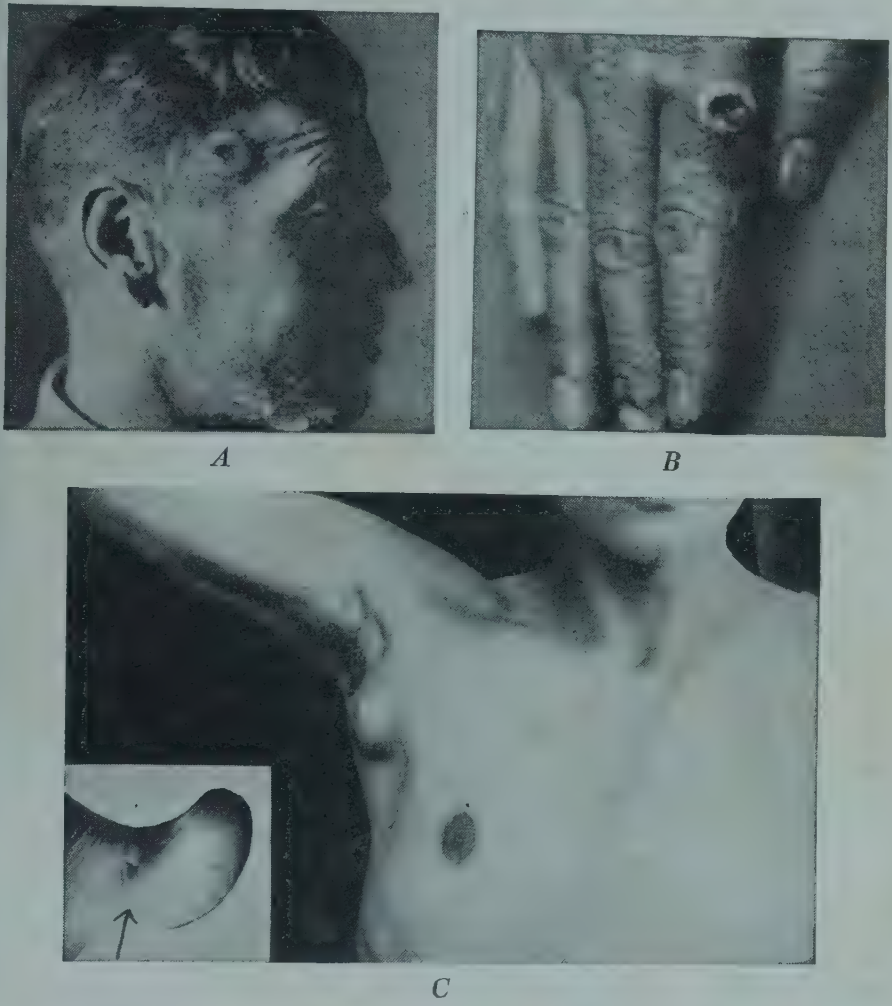

Fig. 361. *A*, Primary ulcer on the temple following bite of fly, *Chrysops discalis,* in Utah. (Dr. Francis, Army Medical Museum, No. 42955.) *B*, Primary ulcer on finger following dressing of wild rabbits; eighteen days after onset. (Dr. Tomas Cajigas, Army Medical Museum, No. 63174.) *C*, Ulcer of thumb and axillary bubo after dressing market rabbits; forty days after onset of illness. (Drs. Brown and Hunter, Army Medical Museum, No. 43709.)

is by handling infected rabbits, as in the marketing of rabbits for food and pelts.[15, 16] Streptomycin appears to be of value in treatment.[17]

GENUS BRUCELLA

Brucella melitensis and two closely related species, *Br. abortus* and *Br. suis* cause the disease *Malta fever* or *undulant fever,* common here as well as in many other parts of the world. The generic name is derived from the discoverer, Bruce, a British scientist who first (1887) found the organism now called *Brucella melitensis* on the island of Malta in the spleens of persons infected by the organisms in goat's milk.

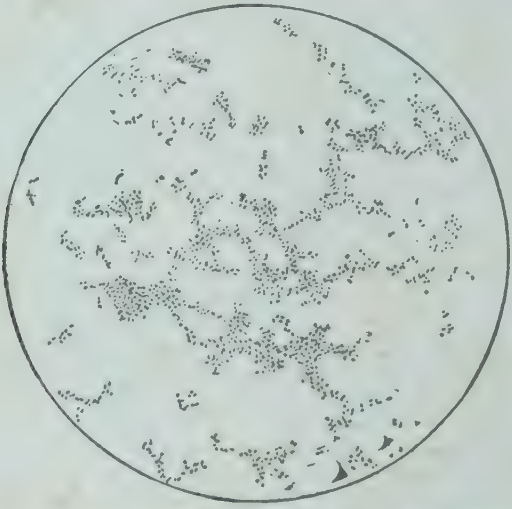

Fig. 362. *Brucella melitensis* (× 900). (Mohler and Traum.) Note the very small size and coccoid appearance of the organisms. In some cultures they appear larger and more distinctly bacillary.

Br. abortus was first known as a bacillus causing abortion in animals. It was discovered by a Danish worker named Bang in 1895 and the organism is still often referred to as Bang's bacillus, and the disease in cattle, Bang's disease.

Br. suis which often infects swine was first observed by Traum in the United States in 1914. Its true relationship to undulant fever was not then known. Only in 1918 did the close relationship of these three organisms become clear through the work of Evans.[18]

Brucellas are small, gram-negative, nonmotile, nonencapsulated, non-sporeforming rods tending to become coccoid (Fig. 362), growing poorly when first isolated from the milk or from the blood or tissues of infected animals or man, and best cultivated in slightly acid (pH 6.8) liver-infusion agar or liver broth or on tryptose agar (see below). *Brucella abortus* will grow well, at first, only in an atmosphere containing 10 per cent carbon dioxide. This peculiarity is shared with a number of other pathogens, especially the *Neisseria.* No carbohydrates are fermented, but they appear to be metabolized with alkaline end-products. All species are somewhat microaerophilic.

A number of improvements have been made in media for the isolation and experimental cultivation of *Brucella.* One is a simple "synthetic" solution of pure chemical compounds.[19] Another is a selective medium for isolation from feces based on tryptose-glucose agar and containing tyrothyricin and sodium azide as agents inhibitory for fecal bacteria.[20] Castaneda has devised a combined fluid medium and agar surface for isolation, the agar being on one side of a bottle, the fluid at the bottom of the bottle. The bottle is turned over daily so as to flood the agar with the fluid and then turned so that the agar is again exposed. This idea should find application in other fields.[21] It should prove useful in connection with the method for isolating *Brucella* organisms from blood clots, as described by West and Borman,[22] and for general bacteriological studies of blood clots.

TABLE XXII

CHARACTERS USED TO DIFFERENTIATE SPECIES OF BRUCELLA

Species	Number of days from commencement of growth during which H_2S is continuously produced	Utilization (not fermentation) of dextrose	Degree of growth vigor on liver-infusion agar containing 1 part in 25,000 of	
			Thionin	Basic Fuchsin
Brucella melitensis . . .	Little or no H_2S	active	good	good
Brucella abortus	4	slight	poor	good
Brucella suis	6–10	active	good	poor

The three organisms in this genus are closely similar but may be differentiated according to the animal they most commonly infect. They may also be distinguished, usually, by the characters given in Table XXII.[23]

Agglutination tests are of little value in differentiation of these species from one another, except by special absorption methods.

One other organism of this genus, *Br. bronchiseptica,* is a good illustration of the difficulty of classifying bacteria (Fig. 363). In its nonfermentation of dextrose and nonliquefaction of gelatin it resembles the brucellas but in many other respects it is almost identical with *Alcaligenes faecalis* (see page 572). It is motile. It has certain factors in common with the organism of whooping cough (*Hemophilus pertussis*) described later in this chapter and it will be discussed there, although it is very different in many other important characteristics. *Br. bronchiseptica* is not related to undulant fever.

Undulant Fever (Brucellosis). This disease is caused by members of the genus *Brucella*. Undulant fever in man is characterized by a long, slow onset with increasing weakness and lassitude. Later there is backache, stiffness of joints, anorexia (loss of appetite), loss of weight, low fever and many other indefinite symptoms, depending on the organs affected by the bacteria and the resistance and reaction of the patient. Exact diagnosis is often impossible unless the organism be isolated from the patient. Remittent attacks of fever of a day's or several days' duration, with severe night sweats, persist over long periods. Rheumatic symptoms are common. Sometimes mild attacks of the disease are diagnosed as influenza or "grippe." The disease is seldom fatal.

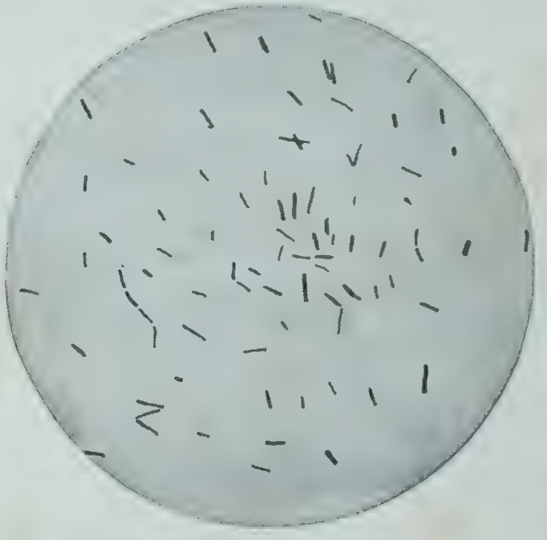

Fig. 363. *Brucella bronchiseptica* (× 900).

Like *Pasteurella tularensis*, the bacilli invade the blood stream in man as in cattle, swine and goats, and localize in foci in the lymphatic system everywhere. They may occur in urine and feces and are fairly resistant to the conditions of the outer world and may survive for some time in soil, etc. In cows and milch goats they tend to localize in the udder and appear in the milk. The organisms are transmitted from animals (cattle, swine, goats) to each other and to man by means of infectious discharges, milk or carcasses. For this reason the disease is found most frequently among farmers, slaughter-house employees and persons in districts where the disease is common in dairy cattle and who drink unpasteurized, uncertified milk. The organisms enter the body from the sources mentioned by almost any portal of entry, but particularly through abrasions of the skin and through the gastro-intestinal tract.[24–29]

Laboratory Diagnosis. The brucellas readily infect guinea pigs if injected into them. Guinea pig injection is, therefore, often used in diagnosis or in attempts to isolate the organisms from blood, or cream, and is sometimes more satisfactory than direct cultivation. However, persons handling infected guinea pigs almost invariably contract the disease, which often disables them for long periods.

If cultures are used in diagnosis, liver or tryptose broth is best for blood while, for cultivation from cream, plates of liver or tryptose agar with a selectively inhibitory dye (crystal violet, 1:500,000) or the Castaneda or other selective media mentioned above to restrain *Streptococcus lactis* and other gram-positive organisms, are sometimes successful. As growth of the brucellas is slow the cultures should be incubated for two weeks unless colonies appear earlier.

The *agglutination test* is a good means of detecting the presence of brucellosis in cattle and in man, especially if a series of tests are made with serum specimens taken at intervals of a week or so after onset of fever, and a distinct *rise in titer* is noted. This *rise in titer* is much more significant in diagnosis than a titer which remains stationary. A rising (or declining) titer in antibodies in any disease indicates an active, present response to infection. A *stationary titer* may represent infection years ago or merely an individual immunological peculiarity not related to infection. A peculiarity of *Brucella* agglutination is the common occurrence of prezones; in which no agglutination occurs in low dilutions of serum, but may appear in high dilutions of the serum. Brucella agglutination tests may also be made with rennet-whey of cows' milk. Herds of dairy and breeding cattle are often examined by means of the agglutination test (often spoken of "Bang testing") to detect latent infection.

An excellent method of detecting agglutinins in *whole* milk is to add a few drops of specially prepared, heavy suspension of stained organisms to about 5 ml. of the milk in a narrow tube, shake well, and let the tube stand upright for a few minutes. If agglutinins are present, the stained antigen will rise to the top and form a distinct layer. [29a]

GENUS HEMOPHILUS

There are several species in this genus, important among which are the "influenza bacillus" (*Hemophilus influenzae,* Fig. 364), the organism of whooping cough (*H. pertussis*), *H. suis,* implicated in swine influenza, and *H. ducreyi,* the cause of a venereal disease called "soft chancre" or chancroid.

The general description of the family fits these organisms perfectly since they are gram-negative, non-sporeforming, aerobic and facultative, very tiny, nonmotile, highly parasitic and sensitive to all but body conditions. These organisms do not, as a rule, ferment dextrose and they are not proteolytic.

Special Nutritive Requirements of the Hemophilic Organisms. Their generic name is derived from the fact that they require not only the usual constituents of infusion media but blood, or certain substances in it, for satisfactory growth, especially when freshly isolated from the body. One substance, for years called an "X factor," is now recognized as a catalase of beef liver or the iron complex called *hemin,* which occurs in the red coloring matter of erythrocytes.

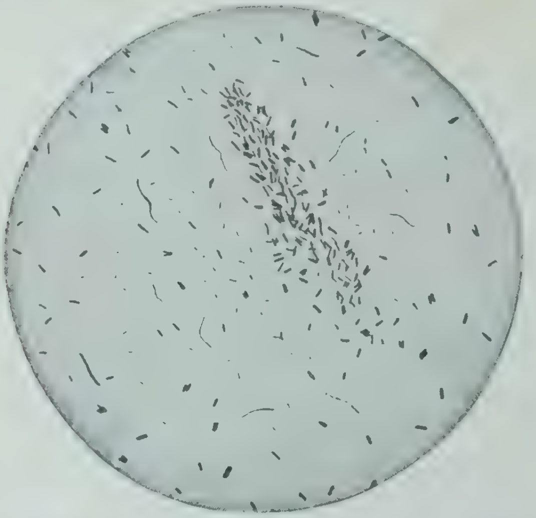

Fig. 364. *Hemophilus influenzae* (× 900).

In addition, most of the species require for growth a substance, originally referred to as "V factor" but now recognized as coenzyme I, extractable from yeast and certain other vegetable cells and also from some bacteria, notably certain strains of staphylococci.[30] The X factor withstands moderate autoclaving, the V factor is easily destroyed by heating. Fresh, whole blood apparently contains both X and V factors in sufficient amounts for hemophilic bacteria. The relationships of these factors to growth of some of the organisms is shown in Table

TABLE XXIII

RELATIONSHIP OF "X" AND "V" FACTORS TO GROWTH OF HEMOPHILUS

Growth in Peptone Broth or Agar	H. influenzae	H. suis	H. pertussis	
			Fresh*	Old†
With neither factor	−	−	−	+
With X factor only	−	−	−	++
With V factor only	−	−	−	++
With X and V factors	+	+	+	++
With whole blood	+	+	+	++

* First isolation from the throat (Phase I).

† After several transfers on artificial medium (Phases II, III, IV).

XXIII (see also section on bacterial respiration, page 289). However, it is possible to cultivate *H. pertussis* on media without blood.[31]

"Satellite Formation." A striking demonstration of the synthesis of coenzyme by staphylococci is seen when a plate containing overheated blood agar (minus the V factor) is inoculated with *H. influenzae all over the surface,* and in *one spot only* with an appropriate strain of *Staphylococcus.* Upon incubation, growth of *H. influenzae* is found to have occurred only in the immediate neighborhood of the *Staphylococcus* colonies.

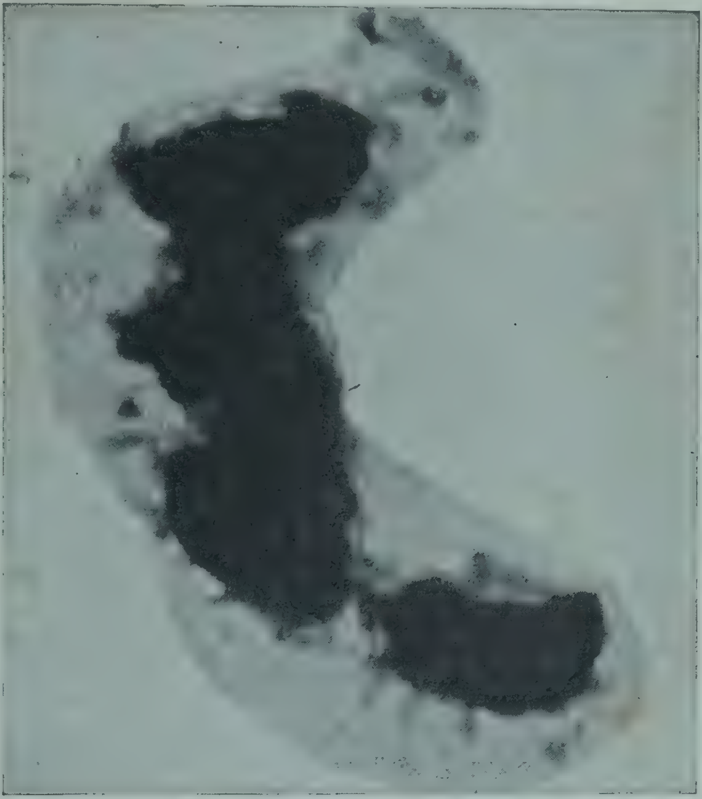

Fig. 365. Electron microscope photograph of *Hemophilus pertussis* (× 50,000). Actually, the bacterium that causes whooping cough is about 0.5 micron in length; this is approximately equivalent to 0.0000195 of an inch. The bacterial body shown is enveloped by a "membrane" or capsule which shows as a lighter surrounding area. The capsule of *H. pertussis* is antigenic. (Dr. Malcolm H. Soule in Therapeutic Notes, May–June, 1943, Parke, Davis & Company.)

Fresh human blood also contains some inhibitory substance which may be removed by heating for ten minutes at 90° C. The blood turns a chocolate brown and, in agar, is referred to as "chocolate agar." This agar is useful for many pathogens, in addition to the genus *Hemophilus,* especially the genera *Neisseria* and *Brucella.*

Hemophilus Influenzae. The pathological relationships of this organism are interesting. It derives its name from the fact that it was formerly thought to cause influenza or colds. It is commonly found in the normal nose and throat. It has no such relation though it may augment the severity of colds and influenza by infecting the already diseased nasal mucosa. It causes a very severe type of laryngotracheitis in infants. It is often found alone in diseased lungs and in cases of meningitis, which it apparently may cause,[32] and in cases of endocarditis (disease of the heart valves). On ordinary rabbit blood agar plates the colonies are 0.5 to 1 mm. in diameter, colorless, and transparent like flattened dewdrops. The colonies are seen with difficulty. *H. influenzae,* like some other bacteria, undergoes the R-S variations. In the R phase it produces very long filaments and swollen cells and may look quite unlike the traditional type of "little bacteria." Some of these filaments are seen in Fig. 364. This sort of morphological variation is seen in many bacteria.

Six serological types (called a, b, c, d, e and f) of influenza bacilli are known. These derive their type specificity from polysaccharides in their capsules and type is determined by a quellung reaction in a manner exactly analogous to pneumococcus typing. Of the six serological types, type b is most frequently pathogenic, often causing fulminating laryngo-tracheitis and meningitis of infants, sinusitis, etc. Untypable strains may also cause such infections.[32, 33]

Hemophilus Pertussis. This, the bacterium causing whooping cough (pertussis) (Fig. 365), was discovered in 1906 by two Belgian workers, Bordet and Gengou. It is sometimes called the Bordet-Gengou bacillus. It is best cultivated *from the body* on agar containing glycerin, potato extract and blood (Bordet-Gengou medium)[*] seeming, *when first isolated,* to require large amounts of both X and V

[*] *Bordet-Gengou medium (Kendrick's modification)*:

Peeled sliced potatoes	500 gm.
Glycerin, U.S.P.	40 ml.
Dist. H_2O	1000 ml.

Boil the potatoes in the glycerin and water until soft. Replace water lost by evaporation. Pass the fluid portion through gauze and allow sediment to settle out. Decant the supernatant liquid.

To 500 ml. of this fluid add:

NaCl (0.75 per cent solution)	1500 ml.
Agar	60 gm.
Proteose peptone	20 gm.

Heat until agar is dissolved, make up to volume, and dispense in desired amounts for storage. Autoclave at 15 pounds' pressure (120° C.) for 25 minutes. When needed, this agar is melted, cooled to 45° C. and blood is added to make a final concentration of at least 15 per cent. Defibrinated blood should be used, which is not more than 72 hours old. Mix blood and agar and pour plates fairly thick (15–20 ml.).

and other factors. However, after a few subcultures it will grow on chocolate agar and even blood-free media. Indeed, semi-synthetic, fluid, blood-free media are used successfully to cultivate fully virulent organisms for the manufacture of vaccines.[34] These media contain yeast extractives and ferrous iron. The organisms may also be cultivated in the yolk-sac of embryonated chicken eggs.[35]

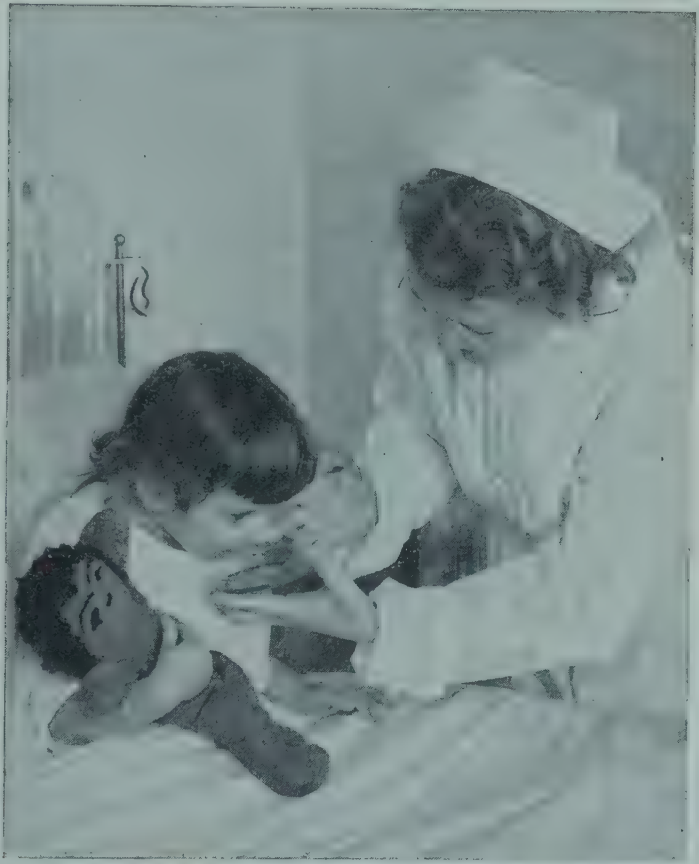

Fig. 366. Use of the cough plate in the diagnosis of pertussis. The Petri dish contains Bordet-Gengou medium and the child is coughing across it. It will be closed and incubated. From cultures such as this Phase I cultures are obtained for the preparation of pertussis vaccines. Photograph by Dr. Franklin H. Top. (Therapeutic Notes, May–June, 1943, Parke, Davis & Company.)

The organisms are coughed up in great numbers during the first three or four weeks of the disease. After the fifth week they are rarely found. They may be caught and cultivated on Petri plates containing Bordet-Gengou agar held in front of the victim's mouth during paroxysms of coughing ("cough plates") (Fig. 366). The colonies appear only after ninety-six hours at 37° C. and look like very tiny pearls or droplets of mercury surrounded by a dark, changed zone of hemolysis (Fig. 367). Identification is made by cultural tests (indol. $NaNO_3$, etc.), and by agglutination with immune rabbit serum. Cough plates are very widely used in the bacteriological diagnosis of pertussis. Nasopharyngeal swabs are also used with excellent results in collecting cultures.[36] By dipping the swabs in penicillin solution after use, but just before streaking the plate, contaminants are eliminated and better results obtained.[37, 37a]

Phases of H. Pertussis. When freshly isolated the organisms are encapsulated, virulent for guinea pigs, mice and rabbits, hemolytic and very fastidious about X and V factors.[38] This is spoken of as Phase I of the organism. After artificial cultivation it loses these properties and degenerates to Phases II, III and IV. It is important to note the development of these phases as one aspect of the general problem of bacterial variation which has a direct bearing on health.

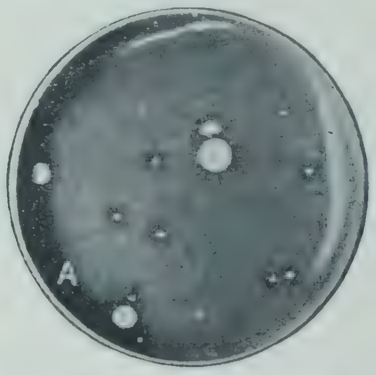

Fig. 367. "Cough plate" from child with pertussis. On Plate *A* the colonies of *Hemophilus pertussis* are very tiny and are surrounded by zones of hemolysis. The large colonies are probably staphylococci. (Sauer and Hambrecht.)

Pertussis Vaccines. Phase I strains of the pertussis organism, killed by appropriate means, when injected into the body stimulate the production of protective antibodies and thus serve as a means of preventing or modifying the severity of whooping cough.[39] If older strains of Phases II, III, or IV, maintained for some time on blood-free media, are used, no protective stimulation is obtained.[40] A good means of testing the potency of vaccines by intracerebral inoculation of mice has been developed.[38] Before these facts were known, many failures occurred in attempts to vaccinate against pertussis. This is illustrative of similar variations in other species of bacteria, notably *S. typhi*, in which Vi strains of the smooth, virulent type are used for bacterins, degenerate rough or Vi-free strains having little or no virtue as bacterins.

The cough in pertussis may be due to a toxin which has been demonstrated by Wood[41] and others[42] to be present in the pertussis bacilli and to appear in filtrates of their cultures. The organisms gain a foothold in the trachea and grow among the cilia of the tracheal epithelial cells, setting up an irritation there (Fig. 368).

It has been found that pertussis antitoxin has a definite protective action for mice and rabbits[42] and that hyper-immune human serum has a definite value in therapy and prophylaxis of pertussis.[37a]

Hemophilus suis is almost identical with *H. influenzae,* differing from typical strains of the latter mainly in failing to produce indol. It is associated with swine influenza, but the relationship is complex. By itself, it causes no disease in swine. Swine influenza is due to a filtrable virus (see page 789). The disease as caused by the virus alone is relatively mild, often passes unnoticed and is seldom fatal. If, as usually occurs under natural conditions, the bacterium happens to accompany the virus when passed in the nasal and oral secretions from one pig to another, the disease caused is often fatal.

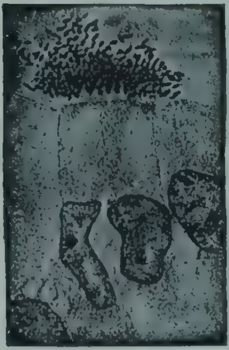

Fig. 368. Whooping cough. Minute bacilli present in masses between cilia of two cells lining the trachea: in the lower part of the picture are seen the nuclei of three epithelial cells of the tracheal lining. Above the nuclei is the cytoplasm of the cells, and on the surface are masses of *Hemophilus pertussis.* (× about 1500.) (Mallory and Horner.)

Bacteria-Virus Complexes. This illustrates a principle of the greatest importance. It has been found that other diseases of obscure etiology represent combined infections of a virus and a bacterium. One long-known example is that bane of the farmer, hog cholera. This disease is primarily one of the blood, is due to a virus and in the uncomplicated form is not excessively fatal. With the added infection of *Salmonella choleraesuis* or *Pasteurella multocida,* it becomes a problem of great economic importance, as the intestinal and lung involvement greatly increases the loss of swine. Unlike swine influenza, the bacterial component of hog cholera produces a dangerous infection by itself in both man and animals. Volkert et al. have shown that the intensity of infection by tubercle bacillin in mice is markedly increased by concurrent infection with influenza *virus* (not influenza *bacilli*).[43]

Hemophilus Ducreyi. This organism is closely related to *H. influenzae* and *H. pertussis* and is morphologically like them, but is shorter, plumper, and with rounded ends, with tendency to bipolar staining. It is not found in the respiratory tract but may be cultivated from the lesions of a venereal disease called "chancroid" or "soft chancre" (as contrasted with the hard chancre, or ulcer, of syphilis). It grows best on soft blood agar[44] or heated, clotted blood or whole defibrinted rabbit's blood, and sometimes forms long filaments. In pathological material the organisms may be seen in clumps or "schools." Contamination with secondary flora in ulcerative lesions makes isolation difficult.

Beeson, Kuhns and others have developed means of diagnosis based on direct smears from lesions and by using tryptose phosphate blood-agar plates heated to 85° C. to "chocolate" the agar. Incubation is in an atmosphere of 10 per cent CO_2. The colonies grow like *H. pertussis* colonies.[43a]

It causes a spreading, and often very destructive, ulceration and gangrene of the external genitalia and adjacent parts and may be seen in smears of pus from such lesions. It is transmitted chiefly by sexual intercourse. Like other members of the genus, it is a fragile organism and soon dies outside the body.

Brucella Bronchiseptica. As shown by Brown[45] and others, this organism occasionally causes a pertussis-like disease of children. It is also a common cause of respiratory disease ("snuffles") of rabbits and other small animals. In several respects it resembles *H. pertussis*, being a gram-negative rod, non-sporeforming and nonencapsulated. Unlike *Hemophilus pertussis,* it grows readily on ordinary agar. It should be classed in the genus *Alcaligenes*. It is actively motile, much larger than *H. pertussis* and grows much more vigorously and is more resistant to unfavorable influences. In these respects it resembles the intestinal organisms. It may be identified by agglutination tests and by other characters shown in Table XXIV.

Eldering and Kendrick[46] described a closely related organism which they named *Bacillus parapertussis*. It has properties of both *H. pertussis* and *Br. bronchiseptica* (Table XXIV). It also is not infrequently found in cases of pertussis-like disease in children. The three organisms show definite antigenic relationships.

THE BACTEROIDEAE

This tribe includes the genera *Bacteroides* and *Fusobacterium*. They have relatively little in common, the *Bacteroides* occurring mainly in the intestine; the *Fusobacterium* in the mouth. Morphologically they are very dissimilar and their cultural requirements are different.

Bacteroides. This genus contains twenty-three species of small rods, most of which are not completely described. They have the general properties of the Parvobacteriaceae. Strictly anaerobic conditions are necessary for growth. They occur in enormous numbers in feces and may be cultivated on ordinary nutrient agar or gelatin but they grow best on blood agar. Their colonies are small, whitish, or grey, often less than 1 mm. in diameter, rarely more than 3 mm. Only one or two species are motile.

In general they appear to be harmless saprophytes. They attack peptones, proteins and carbohydrates, often forming gas. Many produce H_2S. A few species have been found apparently causing such conditions as appendicitis, septicemia, pulmonary abscesses, middle ear infection (otitis media), urinary tract infections, etc. The species so found have been *B. fragilis, B. serpens,* and *B. funduliformis.* They probably are not highly virulent and infections by them may be regarded as accidental occurrences in which they act the role of opportunists.

TABLE XXIV

CULTURAL CHARACTERS DIFFERENTIATING BACILLUS PARAPERTUSSIS FROM RELATED BACTERIA

	NO_3 Reduction	Growth on Plain* Agar	Growth in Plain* Broth	Motility	Indol
Hemophilus pertussis (Phase I)	–	–	–	–	–
Bacillus parapertussis	–	++ (brown pigment)	++ (ropy; brown pigment)	–	–
Brucella bronchiseptica	+	++	++	+	–
Hemophilus influenzae	+	–	–	–	+

* Without blood.

GENUS FUSOBACTERIUM

These are strictly anaerobic organisms, killed by even an hour of exposure to air. They are non-sporeforming, gram-negative, slender, nonbranching rods with pointed tips (Fig. 369). They are common in the normal mouth, and may be seen in smears of material scraped from around the teeth. Their study has been difficult and the genus is not fully known. They require special media, some species growing on blood agar,[47] others on a 10 per cent potato-extract agar containing cysteine.[48] Growth requires about a week at 37° C. Several morphological, fermentative, and antigenic varieties have been differentiated.

The significance of these organisms is not known. As has been shown in the section on spirochetes (page 491), *F. plauti-vincenti* (the type species) is regularly associated with certain species of *Borrelia* in ulcerous conditions of the mouth, often called "trench mouth" or Vincent's angina, but their etiological relation is obscure. The disease may be due to certain deficiencies in the diet. Some species of *Fusobacterium* occur in lung abscesses.

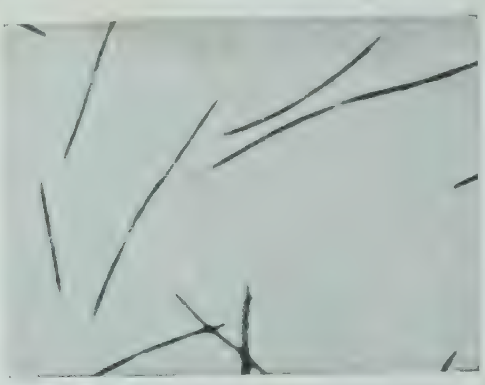

Fig. 369. Fusiform bacteria from the mouth. (× 1000). (Berry and Hine, J. Bact., vol. 34.)

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Chapter 46

THE VIRUSES*

I. GENERAL DESCRIPTION

In 1891 bacteria were the smallest, simplest and lowest forms of life known. Physiologically and structurally they were viewed as the boundary between the living and the inanimate. Investigators of that time felt that they had probed the depths of the mystery of life and discovered its extreme lower limit with respect to size and simplicity of organization. Scientists believed they had reached the "jumping-off place" of smallness. Yet many times the "void and empty" fluids through which their searching lenses swept, teemed with billions of living particles which escaped their vision and their knowledge.

First Demonstration of a Filterable Infectious Agent. In 1892 Iwanowski demonstrated that a disease of the tobacco plant called "tobacco mosaic" could be transmitted to healthy plants by sap from diseased plants after it had been passed through filters of porcelain so fine as to remove completely all bacteria. This discovery was later confirmed and today we realize that Iwanowski opened the door to the *world of the ultramicroscopic,* much as Leeuwenhoek's discoveries opened the door to the world of the microscopic. Today many agents of disease related to the one discovered in 1892 are well known. Because of the invisibility of most of them by ordinary microscopes, and their filterability through fine porcelain filters, they have for years been designated as *filterable* or *ultramicroscopic viruses.* Today it is customary to speak of them merely as *viruses.* It is important to note that our knowledge of them is based on the fact that they produce disease, since all are obligate parasites on *living* cells and none can be cultivated on lifeless media as can bacteria. No saprophytic viruses are known. Viruses present to the biologist, the chemist and the philosopher one of the greatest mysteries: that of the nature of life.

Properties of Viruses. Visibility. The most obvious properties of the viruses may be summarized very briefly. First is their *invisibility with ordinary microscopes.* No one has *with certainty* seen virus particles. They cannot be demonstrated, *beyond quibble,* with any optical apparatus at present extant. Hence the term *ultramicroscopic.* However, it seems probable that certain crystals and certain granules, to be described, are actually viruses. The electron microscope has opened a new field for the investigation of viruses. It is possible to make electronographs showing particles of definite, uniform size and shape under circumstances, and in infectious fluids, which leave the clear impression, and moral assurance, that these particles are indeed the hitherto unseen viruses themselves (Fig. 370).[1] There is, however, a wide gulf between moral assurance and *absolute proof*. Nevertheless, progress in science sometimes results from an expedient assumption; a process which we may refer to as bridging factual hiatus by hypothesis. Such bridges are often built under intellectual verbal fire!

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* *Virus* is a general term meaning, strictly, a slimy substance. The word is restricted in microbiology to the type of disease agents under discussion.

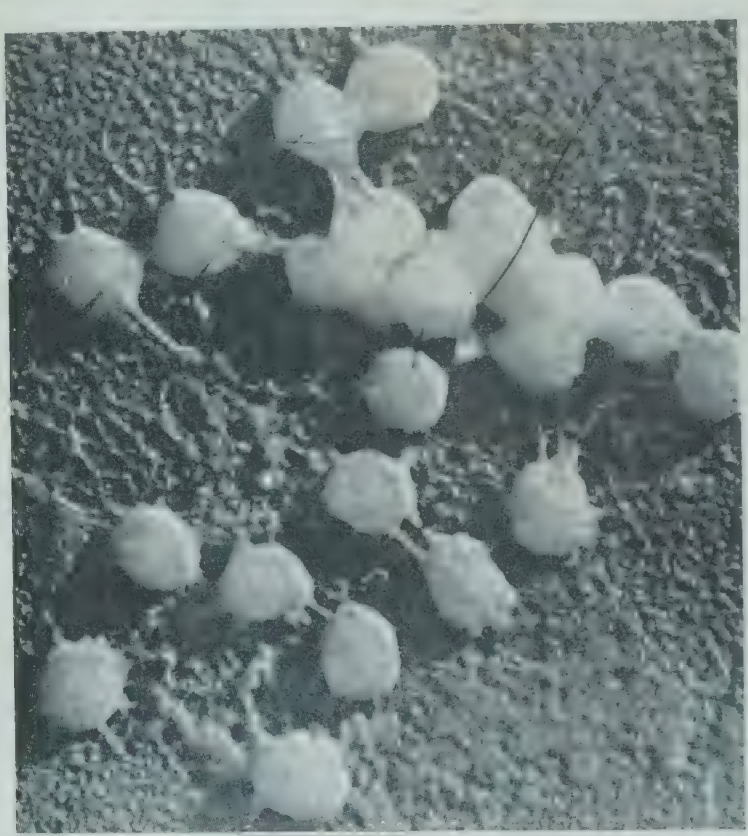

Fig. 370. Electronograph believed to represent one of the larger viruses. Shadowed with 19 mg. of gold at the angle tangent 2-10. 2.3 × 12,400. (Groupé, V., and Rake, G., in J. Bact., vol. 53.)

Elementary Bodies. Several disease agents often classed with viruses (see Chapter 47) are always accompanied by the accumulation in the body tissues, fluids, blood or lymph, of myriads of small refractile granules, smaller than bacteria yet visible with ordinary microscopes. They are often called *elementary bodies*.[2] Their presence and numbers fluctuate with the presence of the disease and the infectivity of the fluid containing them. They may be stained by appropriate methods (Giemsa, etc.). Separated by centrifugation and washed (*supposedly*) free of other substances they retain their infectivity. Their very size and form, which vary greatly, may be characteristic of a given disease. Further, they may be agglutinated by specific immune sera and robbed of their infectivity by such sera. But their actual identity is not known with absolute certainty. Granules of degenerated protein or other matter are often present in material containing viruses, and no sure means of distinction has been described. Viruses tend to attach themselves (by adsorption) to minute particles of various kinds so that one can never be sure, when he sees such granules, whether they are virus particles or merely inert particles on which virus may have been adsorbed. Immune sera might agglutinate such particles and neutralize the virus adherent to them.

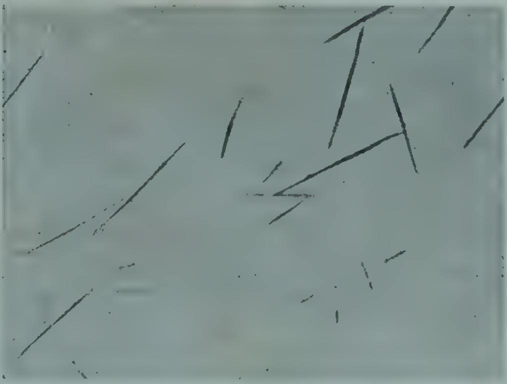

Fig. 371. Crystalline protein from tobacco mosaic. Crystals indicate chemical purity. The chemically pure substance of these crystals is said to be at the same time a living entity. × 400. (W. M. Stanley.)

Many viruses develop in tissues *without* the appearance of demonstrable granules. A cubic centimeter of serum of animals infected with yellow fever may be perfectly limpid to the eye, and perfectly free from any visible abnormal appearance or substance whatever, yet contain sufficient virus so that one millionth part or less of the cubic centimeter can cause a rapidly fatal attack of yellow fever. There is not even any abnormality in refractive index of the serum.[3] This suggests that while some viruses may be large enough to be visible, others approach the size of protein molecules.*

One of the most interesting series of observations in this connection is that of Stanley and others on the crystalline nature of the virus of plant diseases, notably tobacco mosaic disease and "bushy stunt." The crystals are obtained from infectious tissue juice by precipitation methods. The crystals seem to have all of the essential properties of the virus and appear to be nucleoprotein in nature. They are widely accepted, as the actual virus itself (Figs. 371 and 372). No virus of animal disease has been obtained in such form.[1] Electronographs of elementary bodies of vaccinia (Fig. 376) show them to consist of roughly cubical packets each containing five block-like masses of unknown significance.[2] Vaccinia virus is one of the largest (around 200 $\mu\mu$) and most stable of the animal viruses and is not so readily filterable as smaller viruses like those of poliomyelitis, yellow fever or influenza. Its concentration and purification by ultracentrifugation (see page 798) is therefore relatively simple.

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* They may be invisible merely because they have the same refractive index as the fluid in which they are suspended.

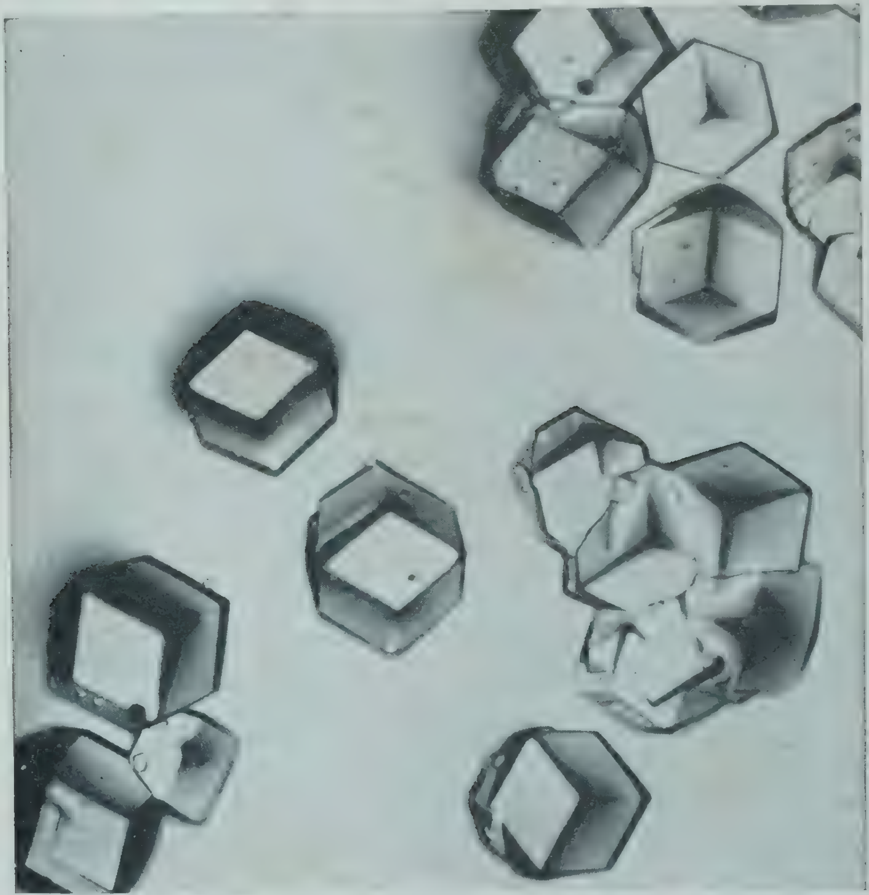

Fig. 372. Crystalline bushy stunt virus. × 224. (Stanley, J. Biol. Chem., vol. 135.)

The electron microscope may be of help in identification of viruses by their morphology, and hence in aiding diagnosis as well as laboratory studies of viruses. Many fine electronographs of viruses are to be found in the modern literature on the subject.[3]

Cultivation. A second characteristic of viruses is their *refusal to multiply* on artificial media, devoid of living cells. No one has cultivated viruses in *nonliving* pabulum. Contrasted with this, however, is the ease with which many viruses multiply in *living cells.* In some instances susceptible animals or plants are the only living material available for propagation of viruses. However, they may be propagated readily in tissue cultures; that is, animal and plant tissue cells can be induced to grow or survive for considerable periods in certain nutrient solutions and these live cells may readily be infected with viruses which then multiply in the cells. One of the most useful and widely used methods of propagating viruses is to inject virus-containing material (blood, tissue juice, saliva, etc.) into living chick embryos (fertile eggs) and incubating the eggs. Technics for doing this have already been described (see Chapter 7, also the monograph by Beveridge and Burnet).[6]

When attempting isolation of viruses from material heavily contaminated with bacteria (*e.g.,* oral and nasal washings, insects, feces, etc.), by means of egg or animal inoculation it has been found helpful to add to the inocula sulfonamide drugs[7] or both penicillin (500 to 1,000 units per ml.) and streptomycin (about 2,000 units per ml.).[8, 9] These agents do not harm the viruses, embryos or animals, yet hold nearly all the bacteria in check. Other workers have used various synthetic bacteriostatic agents, as phenyl mercuric borate 1:50,000.[10] The scientist who devises a means of cultivating any true virus ôn nonliving medium may become the Leeuwenhoek or the Pasteur or Madame Curie of that day!

Filterability. A third distinctive property of the viruses is their *ability to pass through porcelain or other filters which hold back the smallest known bacteria.* This is to be expected of their small size. It is probable, however, that minute size is not the only property which enables them to pass filters, and that forces active at surfaces also play a role. Some viruses are filterable only with difficulty and under special conditions. The agents of vaccinia and rabies are of this type.

Mechanism of Filtration. If the student will recall his studies of physics, he will remember that nearly all very minute particles, when suspended in watery fluids, acquire an electrical charge. Bacteria have negative electrical charges. The particles of material composing porcelain or kieselguhr and similar filters have an opposite charge or at least a charge that is relatively less negative. Thus, when bacteria come into contact with such filters, the two unlike charges result in an attraction and the bacteria are firmly held so that the fluid in which they are suspended passes on through the filter without them. If they are small, and are hurried through the filter under high pressure, they may escape this electrical adsorption and appear in the filtrate. This has caused some workers to regard bacteria as having the ability to pass into growth phases similar to, or identical with, the true viruses. Of course, the filters must be fairly fine in order to bring the bacteria close enough to the walls of the passages or tunnels through the filter so that they come within the very restricted sphere of influence of the mural electrical charges. Channels an inch in diameter would obviously defeat this purpose. Cracks in the filter would act in the same way and are a frequent source of error.

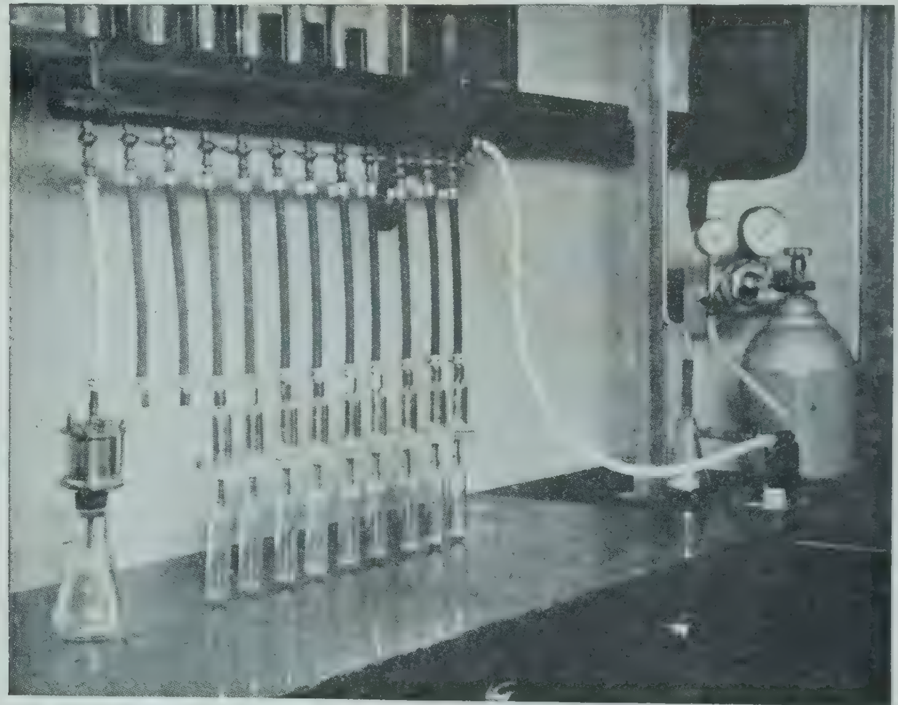

Fig. 373. Series of collodion membrane filters of graded porosities for determining virus particle size. Note the pressure tank and mercury manometer for measuring the pressure applied. The virus suspension is held in the cylinder above each ampoule. The details of the filters are seen in Figure 374. (Bauer and Hughes, J. Gen. Physiol.)

That filterability or nonfilterability of bacteria is, to a great extent, dependent upon electrical forces is made clear by the fact that if filters be composed of material having the same charge as the bacteria (plaster-of-paris), no adsorption of the bacteria occurs and they pass through without difficulty.[11]

Further, if a bacteria-retaining filter be treated with serum, grease, heavy bacterial suspension, protein, or some substance capable of being adsorbed or, in other words, capable of blanketing or neutralizing or rendering ineffective all the electrical charges and other surface forces on the filter particles, then bacteria will pass through without being adsorbed.[12] Thus, with respect to bacteria it seems that it is primarily electrical charges and other surface phenomena, and only secondarily size, that determines filterability through porcelain filters.

This is not so clear when we come to consider viruses. These agents, like other minute particles should, theoretically, possess a negative charge and be adsorbed like bacteria. Contrary to theory, however, viruses are occasionally demonstrated to have positive charges.[13] Still contrary to theory, regardless of charge they all seem to possess the striking property of filterability. Obviously, we are not yet in possession of all the facts regarding filterability of minute organisms.

Size and Filterability. It has been pointed out that the sizes of viruses have a considerable range. This has been proven by filtration through thin membranes of materials of varying degrees of porosity in which surface forces probably play a minor role. Thus, collodion membranes have a certain porosity. The degree of porosity (size and numbers of pores) can be regulated by controlling the density of the solutions of collodion used to prepare the membranes.[14, 15] By measuring the rate of flow of water at given pressures and temperatures through such membranes the diameter of the pores may be calculated. By passing a virus-containing fluid through a *series* of such porous membranes, starting with a coarse and ending with a fine filter, we may estimate the diameter of the virus particles by noting the coarseness of the filters which will pass them and the fineness of the filter required to withhold them (Figs. 373 and 374). The principle is the same as that on which a potato-grader or a gravel-sorter operates.

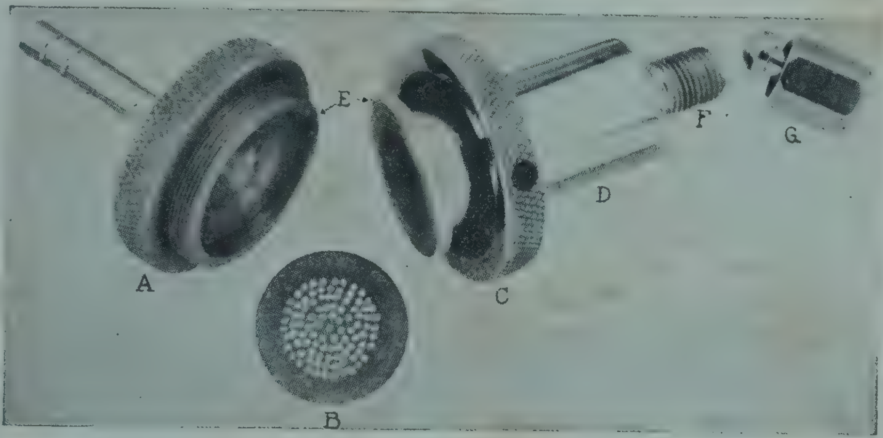

Fig. 374. Detail of the filters shown in Figure 373. The collodion membrane, supported by a disk of filter paper, rests on *B*, which is set in *A*. Cylinder *D* is clamped onto *A* by collar *C*. The suspension is introduced through stem *F*. *G* is a check valve. (After Bauer and Hughes.)

The data obtained by this means are of considerable interest. For

COMPARATIVE SIZES OF VIRUSES

	Molecular weight X 10^{-6} (Particle weight X 6.06 X 10^{17})	Diam. or length X width in mμ
Red blood cells*	173 000 000	7 500
Bacillus prodigiosus*	173 000	750
Rickettsia*	11 100	300
Psittacosis*	8 500	275
Vaccinia*	4 300	225
Myxoma*	4 300	
Canary pox*	4 300	
Pleuro-pneumonia organism*	1 400	150
Pseudo rabies	1 400	
Ectromelia	1 400	
Herpes simplex	1 400	
Rabies fixe′	800	125
Borna disease	800	
Vesicular stomatitis	410	100
Staphylococcus bacteriophage†	300	90
Fowl plague	300	
C_{16} bacteriophage	173	75
Chicken tumor I*	142	70
Rabbit papilloma (Shope)*	47	100 X 14
Tobacco mosaic*	43	280 X 15
Cucumber mosaics 3 and 4*	43	
Gene (Muller's est. of max. size)*	33	125 X 20
Latent mosaic of potato*	26	430 X 9.8
Equine encephalitis	23	38
Megatherium bacteriophage	23	
Rift valley fever	11	30
Tomato bushy stunt*	8	26
Hemocyanin molecule (Busycon)*	6.7	59 X 13.2
Yellow fever	4.3	22
Tobacco ring spot*	3.4	19
Louping ill	2.8	19
Hemocyanin molecule (Octopus)*	2.8	64 X 8
Alfalfa mosaic*	2.1	16.5
Influenza	1.5	15
Poliomyelitis	0.7	12
Staphylococcus bacteriophage†	0.4	10
Foot-and-mouth disease	0.4	
Hemoglobin molecule (Horse)*	0.069	15 X 3
Egg albumin molecule*	0.040	9 X 3

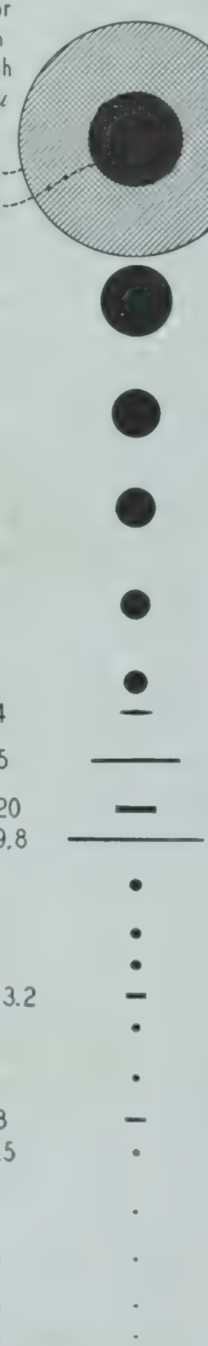

Fig. 375.

example, it is possible to arrange different bacteria, viruses and protein molecules in a more or less accurate comparative series with respect to size as shown in Figure 375.

Demonstration of Viruses. Since viruses are both ultramicroscopic in the ordinary microscopes and noncultivable in inanimate material, it may appear strange that we have any information whatever concerning them. It must be admitted that our knowledge of the viruses is extremely limited. We know nothing of their metabolism, and only those that make themselves conspicuous by producing disease are recognized. Viruses seem to be obligate *intracellular* parasites, deriving their life only from living cells. What information we have, aside from certain physical and chemical data to be described is derived chiefly from methods involving their ability to produce disease and immunity. Thus, we can determine the resistance of viruses to heat, light, disinfectants and other conditions and substances only by subjecting the material containing them to the desired experimental conditions, and then injecting them into some plant or animal which they can infect and observing whether or not disease or immunity results.

An important point, and one that the student should not overlook, is that the injection of an active virus into a susceptible animal, *especially by an abnormal portal of entry,* does not always result in *visible* disease, but that very often in such cases the animal becomes immune and a second, so-called "test infection" is necessary in order to determine whether the first infection had any immunizing effect.

Chemical and Physical Properties of Viruses. In the last few years there have been intensive investigations of the physical and chemical properties of viruses.[16, 17] The stability of plant viruses, permitting them to be precipitated out of suspensions by chemical means (such as 0.4 per cent saturation with ammonium sulfate), adsorbed on celite or other finely divided material like kaolin or bone charcoal, eluted, reprecipitated, and otherwise manipulated for purification and concentration of workable quantities, without undue loss of activity, has made them a valuable subject for experimental research.

Plant Viruses. Stanley and his colleagues have obtained an extensive knowledge of plant viruses by means of highly purified preparations. Tobacco-mosaic virus, one of the most widely studied plant viruses, occurs as needle-shaped liquid crystals (the paracrystalline state) which have been subjected to a variety of physical and chemical studies. It has been found, for example, that the crystals are doubly refractive, and possess positive electrical birefringence. The dimensions, as determined by calculation from sedimentation rates, specific viscosity and other physical data, are about $15\mu\mu$ by 120 to $280\mu\mu$. Electron microscope findings give approximately the same figures. X-ray analyses yield information on molecular arrangements, etc.

Chemically, the virus appears to consist entirely of nucleic acid of the yeast type and amino acids, with neither lipoids nor carbohydrates present. Amino acids in the nucleic acid of the virus include arginine, cysteine, glutamic acid, tryptophane, leucine, tyrosine and several others. Variations in the virus analogous to bacterial variations, and resulting in the production of new strains, are associated with definite changes in the amino acid content. These strains are immunologically distinct.

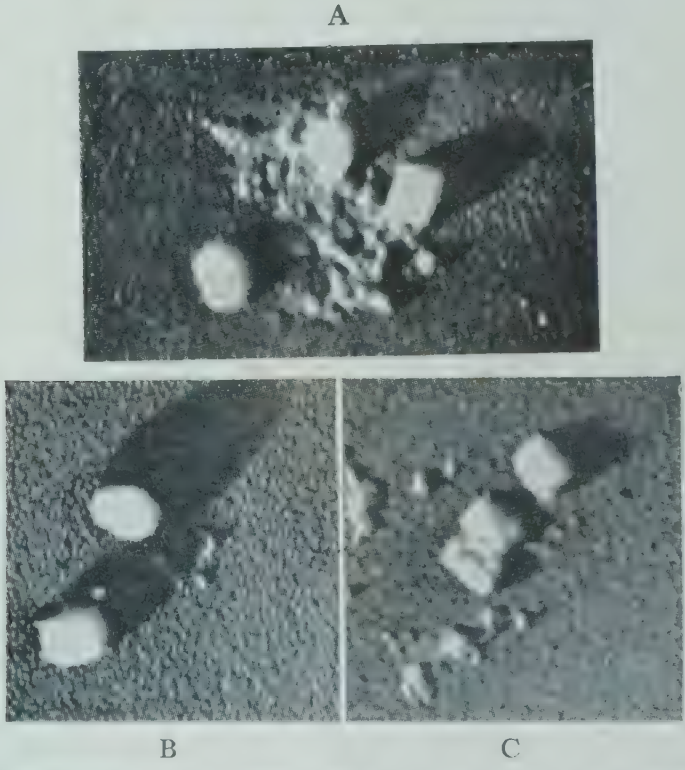

Fig. 376. Vaccinia virus, shadowed with 22.1 mg. gold at the angle tangent 2/11.82. × 24,800. (Nagler, F. P. O., and Rake, G., in J. Bact., vol. 55.)

Animal Viruses. These viruses are less stable than that of tobacco mosaic, especially viruses like those of influenza, yellow fever, and poliomyelitis. They are soon destroyed by the chemical and physical treatments used to purify and concentrate plant viruses. However, animal viruses have been purified and concentrated to a great extent, although none, with the possible exception of poliomyelitis virus, has been crystallized.

The most useful method of purifying and concentrating animal viruses for experimental purposes has been by means of the high speed centrifuge, driven by air turbines, on air bearings, in a vacuum. The process of purification and concentration consists in centrifuging first at relatively low speeds representing about 10,000 times gravitational force (10,000 G), discarding the relatively coarse sedimented impurities and then centrifuging the supernatant at a speed producing a centrifugal pressure equal to about 50,000 G. The sediment thus obtained contains much virus and is resuspended. The suspension is again subjected to alternate low and high speed centrifugation until a material with uniform specific gravity and other physical properties (*i.e.*, not a mixture) results. Such material may be subjected to various physical and chemical studies such as determinations of molecular weight, chemical composition, etc.

All viruses, plant and animal, so far isolated in this manner have been found to be proteins of very large molecular size and weight. For example, from sedimentation constants, diffusion rates, and other physical data the molecular weight of tobacco mosaic virus is estimated to be 43 million, that of influenza A virus about 650,000. Filtration and electron microscope experiments yield data concerning virus particle size which agree, roughly, with the molecular weight data obtained with ultracentrifuge methods. The diameter of the influenza virus particle seems to be about $100\mu\mu$.

Many other chemical and physical devices have been used in studies of viruses. For example, suspensions of some viruses are slightly opalescent due to scattering of light by the particles. From measurements of light absorption by such suspensions the molecular weight and size of the particles may be calculated.[18] The nucleic acids and carbohydrates and other components of several viruses have been studied, both quantitatively and qualitatively. Both ribonucleic and desoxyribonucleic acids are present in influenza virus.[19] By means of electrophoresis it has been shown that the natural virus of yellow fever has different electrical properties from the modified (neurotropic) yellow fever virus.[20] The practical use of some of these methods in attempting to prepare a purified vaccine to Japanese B encephalitis virus has been described by Duffy and Stanley.[21]

It is important for the student to remember that a great difficulty in all such studies of the physical and chemical properties of viruses, especially animal (as contrasted with plant) viruses is in being *certain* that the virus material is *really free* from adsorbed proteins of the host cells. *All data* in this field unfortunately must, for the present, be viewed in this light.[22]

Resistance of Viruses. Studies on the resistance of viruses to various physical and chemical agents have yielded important information. In general animal viruses are killed in a few minutes by temperatures like that of pasteurization or even as low as 56° C. They are highly resistant to freezing and will remain alive at —76° C. for a year or longer.[23] They are readily preserved by desiccation *in vacuo* after rapid freezing (*Lyophil*). Disinfectants like phenol, formaldehyde and iodine quickly inactivate them. Ultraviolet light is rapidly destructive. Sulfonamide drugs, penicillin and streptomycin are usually ineffective in treating virus infections and are harmless in vitro to viruses, probably because the viruses multiply inside of tissue cells and thus escape the action of the drugs. The status of chemotherapy in virus and rickettsial diseases has been summarized by Rivers.[24]

Antigenic Analysis of Viruses. Antigenic analyses of viruses are of particular interest and importance. Tobacco mosaic virus, for example, seems to consist of but a single protein antigen, producing one antibody. On the other hand, animal viruses in general seem to consist of two or more antigens: the virus protein, which stimulates neutralizing antibody production, and a soluble antigen or mixture of antigens which appear to be separate substances engendering the production of agglutinins, precipitins, etc. Vaccinia virus, for example, seems to consist of several antigenic substances, possibly five, corresponding to the structure shown by the electron microscope.[25, 26]

Complement fixation methods, first demonstrated in the first human viral disease, yellow fever,[27] are now being found useful for the antigenic study of a number of viral diseases. The antigen is derived from infected animal or plant tissues prepared in various ways, or from infected chick embryos. The serum containing complement-fixing antibodies is derived from recovered animals or man. The serum of patients in the *acute* phase of some virus diseases, as yellow fever, contains sufficient antigen to give a positive reaction in the presence of immune serum. This is of importance as it is one of the few instances in which complement fixation may be used for early diagnosis.[28] Preparation of antigens for complement fixation studies of neurotropic virus diseases has been described by DeBoer and Cox and others.[29, 30]

Practical use of some of these methods is illustrated by studies of Japanese B encephalitis in Okinawa[30] during the recent war, and yellow fever in Brazil a few years ago.[31]

Other means of studying antigenic differences between viruses are (1) *animal protection* by specific immune sera, and (2) *red-cell agglutination tests*. The technical details are described farther on. By these means it has been possible to demonstrate the existence of whole spectra of closely related but antigenically distinct viruses, somewhat analogous to the Kauffmann-White system for *Salmonella*. Thus, although there are several distinct kinds of virus (smallpox, poliomyelitis, influenza) new data derived from protection and red-cell agglutination tests make it clear that each of these is, to use an analogy with bacteria, a sort of type species and that there are numerous other closely related but unclassified viruses. Some have recently been isolated. In the case of others no isolation has been made but the existence of a virus is pretty clearly demonstrable on clinical and epidemiological grounds. For example, related to the well-defined influenza A and B viruses are many others causing clinical influenza and similar diseases but not easily isolated or classified. Some of these cases are undoubtedly due to Newcastle virus, the cause of a fatal disease in poultry.[32] There may be thirty or more "varieties" of poliomyelitis-like viruses and a large number of related viruses causing encephalitis (inflammation of the brain) in man and animals.[33, 34] An example of how remote and obscure some of these may be is seen in the isolation of a hitherto unknown neurotropic (affecting brain and nerves) virus found in certain mosquitoes in Uganda, Africa.[35] Some of these neurotropic and other virus diseases have been discussed by Meyer and Eddie.[36]

The more viruses are investigated antigenically, the more complex the taxonomical and epidemiological picture becomes. In the 1948 edition of the "Manual of Determinative Bacteriology" an interesting attempt has been made to classify and name the viruses, but it is evident that for some time the science of virology will outstrip the systematist.

Relation of Antibodies to Immunity in Viral Infections. We have seen that viruses are obligate intracellular parasites. If a virus, on entry to the body, immediately enters tissue cells it is protected from antibodies of the blood. For example, if a neurotropic virus such as that of rabies is introduced directly into nerve tissue, even though a high titer of serum antibodies is demonstrable at the time, infection will occur. On the other hand, if the virus, as yellow fever virus, must be carried by blood to the susceptible tissues, antibodies present in the blood will "neutralize" the virus before it gets to the susceptible tissues, and infection will not occur. These facts are of practical importance since, as pointed out by Francis, if blood transmission of a virus is essential to infection, production of antibodies in the blood stream by vaccines and antiviral serums would seem to offer an effective means of prophylaxis. This is well illustrated by vaccination against yellow fever and the use of antibodies to prevent measles. "Antibodies are effective in virus immunity according to the invasive mechanism involved, the type of parasitism and the availability of antibody at the portal of entry."[37] Similar principles may well apply in rickettsial and some other infections such as cholera in which the organisms are confined to the bowel (see page 327).

Malnutritional Resistance to Viruses. It is clear that the multiplication of viruses is dependent on the life of the host cells and on their metabolic processes. It might be expected that if the metabolism of host cells were disordered or impaired, an infecting virus might cease to multiply or at least be held in check. This seems to be the case in several instances. For example, resistance of mice to poliomyelitis virus is enhanced by thiamine deficiency. Mere partial starvation on a complete diet has a similar but not so marked an effect. Poorly nourished chicks are almost completely resistant to a virus producing a cancer-like (sarcoma) disease of fowls; in mice, certain amino-acid and other food deficiencies increase resistance to tumor-producing viruses; undernourished rabbits are less susceptible to vaccinia virus than well-fed animals. Other examples are known. How far it is safe to generalize on this basis is difficult to state, but the phenomenon is deserving of extensive investigation.[38, 39, 40]

Another phase of this problem has been attacked through a direct experimental interference with the host cell respiratory enzymes by means of a dye (malachite green). This dye is known to block the action of coenzyme. Takahashi has shown that by using low concentrations of the dye in tissue cultures of tobacco plants, multiplication of the virus was greatly reduced without harming the plant cells. The possibilities of the method in virus studies, employing other dyes, etc., which specifically block other enzymes, are obvious. The possible value of such substances in the chemotherapy of viral diseases is also clear.[41]

Mutual Interference by Viruses. As will be detailed later, many viruses have a high degree of host-species specificity, *i.e.*, they can infect only certain species of plant or animals. They also exhibit a marked specific affinity for certain tissues of the specific host. The phenomenon of virus interference described below suggests that many viruses are restricted to certain cells of special tissues of a specific host. They may even be restricted to certain receptors or parts of those cells. At least it is clear that, if certain cells are preempted by one virus, another having an affinity for the same cells is excluded and cannot infect. This is mutual interference. Presumably the whole surface of *all* cells is not covered by the first virus. Therefore the second virus could infect, were it not restricted to certain parts of certain cell surfaces for its entry. For example, once certain plants become infected with the virus of tobacco mosaic they cannot be infected with a closely related variant of that virus. If monkeys are infected with a neurotropic variant of the normal yellow fever virus, they are immediately resistant to the normal yellow fever virus. If rabbits are infected with fibroma virus they are immediately wholly resistant to myxoma virus. In simultaneous inoculations with two viruses, one preempts or infects; the other fails. Even inactive (irradiated) virus of influenza will preempt the susceptible cell receptors and prevent infection by live influenza virus. Virus of canine distemper modified by passage through ferrets, and of low virulence for dogs and foxes, will eliminate fully-virulent distemper virus, not only if injected first, but even if given several days after the virulent virus.[42–44]

The value of these observations in relation to the prevention and cure of viral infections is obvious. It may be, for example, that the true effect of Pasteur treatment for rabies lies not in development of antibodies but in blocking susceptible cells with a dead or attenuated virus so that the active virus from the bite of a rabid animal cannot take hold.

That the mere presence of one virus is not necessarily *always* sufficient to eliminate others, however, is made clear by the work of Syverton who showed that it is possible in certain circumstances to superinfect single host cells with a variety of viruses, both closely and less closely related.[45]

As stated by Francis "The fundamental interactions which determine selective localization and pathologic injury are not known. Conversely, the phenomena which differentiate naturally susceptible tissues and species from the resistant are essentially unknown."[37]

Mutation of Viruses. Viruses are often said to mutate. This term implies an heredity mechanism the existence of which is not demonstrated. However, viruses do change very strikingly. Classical examples are the relationship of smallpox to vaccinia and the modification of canine rabies virus by rabbit passage to produce the Pasteur "treatment." Other examples are seen in the change of the pantropic virus of yellow fever first to the neurotropic type by mouse-brain passage and later, by means of tissue culture and egg-to-egg passage, to the harmless type used so successfully for vaccination against yellow fever.[46] Green has shown that the lethal virus of canine distemper may be inoculated into ferrets and that after a number of ferret-to-ferret passages the virus no longer kills dogs and foxes but can be successfully used as a vaccine of enormous value to fox ranchers and others.[47] An even more striking alteration is seen in the complete transformation of the relatively benign fibroma virus of rabbits to the highly malignant and fatal myxoma virus (see page 830).

The value of animal (rabbit) passage in so attenuating hog cholera virus that it immunizes without producing perceptible disease has been shown by Baker[48] and by Koprowski, James and Cox.[49]

It is evident that animal viruses possess (1) a *host specificity* that may be altered with or *without loss of virulence* by adaptation to another animal; (2) *tissue specificity* that may be changed by constant contact with different tissues of the same host; and (3) a *cell specificity* and even (4) a *specificity for portions of certain cells.* Green speaks of changes in host and tissue affinities as zoologic and histologic modifications of virus.

Are Viruses Alive? The question of the living nature of the viruses is an extremely interesting one. Here we have bodies not certainly visible, of such size that they can consist of only relatively few protein molecules, noncultivable, having no detectable metabolism yet capable of enormous and rapid multiplication, capable of existing in clear fluids in extremely high concentration yet often undemonstrable except by pathologic activity. Are we dealing with living structures or with minute non-living particles or with fluids? Could a fluid be alive? If viruses cannot be made to metabolize anything outside of living cells, how do we know that viruses actually exist as separate living, autonomous entities? Might it not be that certain cells, stimulated in a certain way, give off an enzyme-like substance capable of stimulating other cells to give off the same sort of lethal substance? Yet this stimulus must always come from outside. It never starts spontaneously. However, we have seen in Chapter 8 that a substance (ribonucleic acid) applied to the exterior of certain live pneumococci stimulates those cells to continue to produce more of the same substance. Actually, there are no final answers to these questions at present available. All we can say is that some viruses may exist as living bodies, like elementary bodies; some as crystals which are not living as the word is ordinarily defined by biologists; and some as mere aggregations of a few molecules of protein. Their true nature or structure remains a mystery.

The fact that immunity results when animals recover from virus infections[50] suggests that the viruses are antigenic, and therefore protein, in composition. Chemical data support this view. It has been intimated that viruses may be related to the extremely primitive, subvital bodies which might have preceded cellular organisms. The fact that we know little of the structure or metabolism of viruses is all that can be advanced in support of this suggestion. The fact that viruses are strict parasites on higher cells suggests that they may have developed only after the evolution of cellular life. Viruses causing diseases of man and animals are generally assumed to be alive.

Origin of Viruses. According to Burnet "We can state dogmatically that there is no evidence whatever that any virus, whether its host be animal, plant or bacterium, arises de novo. Every virus particle, like any other organism, derives by genetic descent from some similar particle . . ." This is a widely accepted view.[51] Also widely held is the view that viruses probably are derived from larger parasites (bacteria, rickettsiae) by a process of degenerative or adaptive evolution.

It is interesting to find a somewhat modified view in respect to certain plant viruses. DuBuy and Woods present data suggesting that, in plants, the antecedent particles from which viruses originate are constituents of chondriosomes or *normal* portions of cells. These portions, under ordinary conditions, control certain variegations in plants and *remain within the cells.* Under certain other conditions, it is suggested, they can *pass from one cell to another* and set up *self-perpetuating variegations* which closely simulate virus infections. Evidence is presented to show that the chondriosomes undergo mutation and that these mutants represent virus particles.[52] The hypothesis is an interesting one of basic importance and should be further studied. The question involves the entire problem of the nature of viruses and the mechanism of mutation, plasma-genes, etc.

Parasitic Status of Viruses. A very interesting suggestion concerning the origin and nature of viruses has been advanced by Green[53] and elaborated by Laidlaw.[54] We have seen that bacteria, when they become parasitic, lose their rugged independence of living hosts and lose some of their metabolic or synthetic powers, and their resistance to the outer world, through long generations of life in a sheltered environment where much of their food is prepared for them. Such bacteria may derive from their host only simple substances such as constituents of saliva or feces or they may take certain nutrient materials from the blood, or very complicated substances like vitamins, depending on how high a degree of parasitism (or dependence on host) they have evolved. They do not as a rule, however, require to live *inside* the host cells. They are extracellular parasites. Advancing a step further, we may imagine that certain bacteria have undergone such permanent changes in size and metabolism that they can actually enter the host cell and become an intracellular parasite, capable of living only there, perhaps at the expense of the proteins and other essential constituents of the cell, and losing more and more of their own synthetic and other physiologic powers.* As these powers are lost, through indolence and lack of exercise thereof, size diminishes until nothing remains but a bit of substance, probably protein in nature, ultramicroscopic in size, able to pass through the finest filters, capable only of reproducing its kind by division, and capable of life only if furnished with the cell-substances of some particular animal or plant to which it has become adapted. It would, "as it were, live a borrowed life, truly the supreme summit of parasitism."† Such a parasite would possess, as characteristic of itself, only the protein transmitting the specific properties of the virus. It would resemble the gene of the geneticists, independent and incarnate!

So viruses may be supposed to have originated through the development of parasitism to its ultimate perfection; an evolutionary process of a highly successful sort if we regard first-rate parasites as the goal of Nature; an involutionary or degenerative trend if we regard the independent, self-supporting creature as the universal ideal. The latter, carried to its logical, nonparasitic extreme could be only of the autotrophic type.

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* Indeed, just such visible but extremely minute, intracellular, bacterium- and virus-like parasites are well known and constitute an important group of organisms known as rickettsias, which are discussed in Chapter 49.

† Laidlaw, "Virus Diseases and Viruses," 1939. By permission of The Macmillan Company.

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Chapter 47

THE VIRUSES

II. VIRAL DISEASES

Numerous diseases of human beings are caused by viruses, among them measles, rabies, smallpox, yellow fever, dengue fever, anterior poliomyelitis (infantile paralysis), encephalitis (inflammation of the brain), chickenpox, herpes simplex ("fever blister"), warts, common cold and influenza. Many diseases of farm and wild animals are also caused by viruses; common ones are hog cholera, fox distemper, swine influenza, foot and mouth disease, chicken cancer and fowl plague. Several viral diseases of animals also affect man: equinine encephalomyelitis, yellow fever, rabies, psittacosis or "parrot fever." "Mosaic diseases" of tobacco and tomatoes, and certain wilts and blights of other plants are also viral diseases.

Classification of Viruses. In the 1948 edition of "Bergey's Manual" the viruses are included as the order Virales, divided into three suborders named Phagineae (the bacterial virus or bacteriophage); Phytophagineae, the viruses, like tobacco mosaic, causing plant diseases; Zoophagineae, the viruses, like yellow fever, causing diseases of man and animals. The appropriateness of this classification will doubtless be fully discussed in the literature on the subject in the next few years. A classification of the viruses of animal disease may be based on the types of tissues or organs principally affected. Another sort of classification could be based on mode of transmission and another on type of disease caused. Such a list may be constructed as shown in Table XXV.

Any such classification, while convenient for purposes of discussion, is highly arbitrary and can not take into consideration the fact that, although certain tissues may be those visibly or principally affected, other tissues may also be involved so that another grouping, which we might call *pantropic,* would include many of those classed above. For example, psittacosis becomes generalized and affects liver, spleen and gastro-intestinal tract, but this differs in different animals; smallpox can become generalized, and so on. Viruses may become modified and cause atypical or entirely different sorts of disease. One is at a loss to place some viruses, such as that of mumps and of fowl plague in the table. Further, the tissue affinities of some viruses can change completely.[1–5]

Intracellular Inclusions in Viral Diseases. When viruses invade animal tissue cells, they often produce changes in the cells which can be recognized with the microscope. Certain granules, or groups of granules, develop *inside* the cells. These are often quite characteristic in appearance and staining properties and may enable a skilled pathologist to diagnose the disease from the appearance of these granules or bodies. Since the bodies are included in the cells they are spoken of as *intracellular inclusions*. Bacterial and rickettsial infections do not regularly cause such definite inclusions to form. Some of these inclusion bodies are typically present only in the cytoplasm and are called *cytoplasmic inclusions* (Fig. 377). Others are found only inside the nucleus and are called *intranuclear inclusions* (Fig. 378).

TABLE XXV

CLASSIFICATION OF SOME ANIMAL VIRUSES

Group	Tissues Principally and Visibly Affected*	Diseases Caused	Mode of Transmission*
Dermotropic	Skin; mucous membranes of nose and mouth	Various pox-like diseases (smallpox, fowl pox), herpes, warts, measles	Close contact; probably sputum, fomites
Neurotropic	Nervous tissues	Poliomyelitis	Unknown. Flies? Feces?
		Rabies	Bites of animals
		Various encephalitides	Mosquitoes?
Pneumotropic	Respiratory tract	Influenza Psittacosis Pneumonitis	Nasal and oral discharges
Viscerotropic	Various internal organs	Yellow fever	Mosquitoes
		Dengue	Mosquitoes
		Louping ill	Ticks
		Rift Valley fever	Mosquitoes
Neoplastic	Various	Fowl sarcoma	Unknown
		Fibroma of rabbits	Unknown
		Myxoma of rabbits	Unknown
		Fowl leukemia	Unknown

* Under natural conditions.

The nature of these inclusions is obscure. Some workers regard them as intracellular colonies of the virus particles. Others believe them to be merely changed cellular material or detritus (remains of broken down tissue), representing damage by the virus but not the virus itself. Probably in some cases, as in lymphogranuloma, psittacosis and related diseases, the granules actually are virus particles. In other instances, as yellow fever, the question is unsettled. Yellow fever inclusion bodies are of two types: intranuclear[6] and intracytoplasmic.[7] The cytoplasmic bodies of yellow fever are cellular detritus (Fig. 377).

Rivers' Postulates in Viral Diseases. Viruses were unknown at the time of Koch, so that he failed to take these invisible, noncultivable agents of disease into consideration in stating the criteria by which the causal relationship of a pathogen to a disease might be determined.

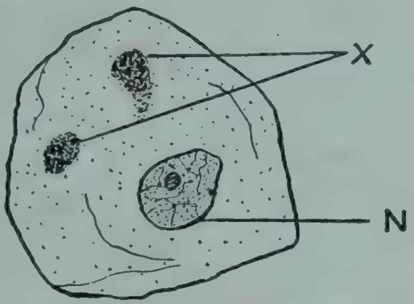

Fig. 377. One type of cytoplasmic inclusion bodies shown at *X*. Nucleus at *N*.

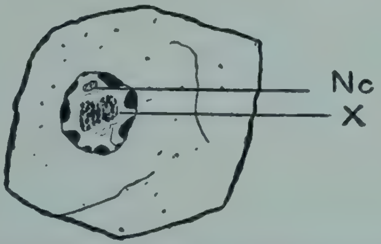

Fig. 378. Intranuclear inclusion bodies in yellow fever. *Nc*, Nucleolus; *X*, inclusion bodies. Note the peculiar lobulations at the nucleus wall. (Redrawn from Cowdry and Kitchen.)

Rivers, in 1937,[8] outlined criteria similar to Koch's postulates, which might apply in the case of viruses. Essentially these are as follows:

1. The virus must be present in the host cells showing the specific lesions, at the time of the disease, or in the blood or other body fluids.
2. Filtrates of the infectious material (blood, etc., or tissue triturates) shown not to contain bacteria or other visible or cultivable organisms, must produce the disease or its counterpart in appropriate animals or plants.
3. Similar filtrates from such animals or plants must transmit the disease.

Immunological and histological studies should confirm the specificity and identity of the diseases caused, but this is not always possible. Some of the many difficulties in such demonstrations lie in the fact that

(1) susceptible animals are not always known or available; (2) many animals have latent virus infections of their own which become active in laboratory experiments and may produce lesions and symptoms closely simulating those of the virus under investigation;[9] (3) latent viruses may become mixed with the virus injected;[10] (4) immunological cross reactions may occur, or the virus injected may cause little immunity while a latent virus may stir up its own immunity; (5) in some instances a virus requires the synergistic aid of a bacterium or another virus to cause typical disease.[11, 12]

Some Viral Diseases. In dealing with bacterial diseases we find each to present problems peculiar to that infection yet to exhibit characters illustrative of certain phenomena common to many bacterial infections. So, also, we encounter a great variety of problems in viral diseases, related to portals of entry, modes of transmission, insect vectors, immunity and the like, many of which are common to bacterial phenomena. We should give some attention to these at this point.

One of the viral diseases of man and animals which has been most intensively investigated is yellow fever. It was the first virus of human disease to be discovered. The facts concerning yellow fever and yellow fever virus served to point the way to many things which have been discovered in connection with other viruses. In addition, the history of the development of our knowledge of yellow fever is of some interest.

YELLOW FEVER

This is often cited as an example of a viscerotropic virus because the normal form affects chiefly the liver and other viscera. Yellow fever is endemic* only in certain tropical regions. The disease was probably brought from Africa to the western hemisphere by Portuguese and Spanish explorers and slave traders as early as the year 1500. It was later distributed through all the Caribbean Islands and Central and South America, taking a terrific toll of life wherever it appeared. For centuries the "yellow jack," as it was called, made life in the tropics a matter of extreme peril. No one knew how it spread or how to avoid it.[13] Hindle states, "In the 90's of the last century the crews of ships going to South and Central American ports were to a large extent shanghaied [kidnapped and forced into service] and this captain himself obtained command at the early age of twenty-one by volunteering to take the ship to Santos. On this particular voyage all except three of a crew of twenty-one died of yellow fever, so their fears were fully realized."[14] When the French tried to build a canal through Panama, the laborers died in such numbers from yellow fever, malaria and dysentery, that the attempt was abandoned. During the Spanish-American War, many hundreds of American soldiers died of the disease in Cuba, even after a general campaign of "sanitation" had been carried out.

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* Constantly present among the human population.

The military authorities finally appointed a yellow fever commission composed of James Carroll, Jesse W. Lazear and Aristides Agramonte under the direction of Walter Reed (Fig. 379). These men, armed only with the very little evidence then available, set out with a grim determination to solve the problem of yellow fever.

Fig. 379. Bust of Walter Reed unveiled during ceremonies at New York University, in 1948. In the words of President Eliot of Harvard, as engraved on the stone which marks Reed's grave in Arlington: "He gave to man control over that dreadful scourge, yellow fever." (Photo courtesy The Hall of Fame, New York University.)

Many different kinds of bacteria had been blamed as the cause of yellow fever. Reed first made a thorough bacteriological investigation testing each of Koch's postulates, and came to the conclusion that the disease was not due to bacterial infection.

A generally held idea was that fomites (bedding, clothing, dishes, etc.) contaminated with the vomitus or feces of victims of yellow fever were very dangerous vectors of the disease-producing agent, whatever it might be.

Another idea, advanced by Carlos Finlay (Fig. 380), a Cuban scientist of Scottish descent, was that yellow fever was transmitted by the bite of *Aedes aegypti* mosquitoes. Finlay's idea was treated rather contemptuously by many wise men of the times. It seemed preposterous!

Walter Reed and his colleagues arranged experiments to test these two hypotheses. They built a little, screened house, divided into two halves. They put beds, chairs and other simple furnishings on each side. In one compartment they piled blankets, sheets, bedding, clothes, and other fomites of men who had died of yellow fever. No mosquitoes were allowed in this compartment, but a number of the insects, known to have bitten yellow fever patients, were placed in the other compartment which was clean and free from fomites.

Fig. 380. Carlos J. Finlay.

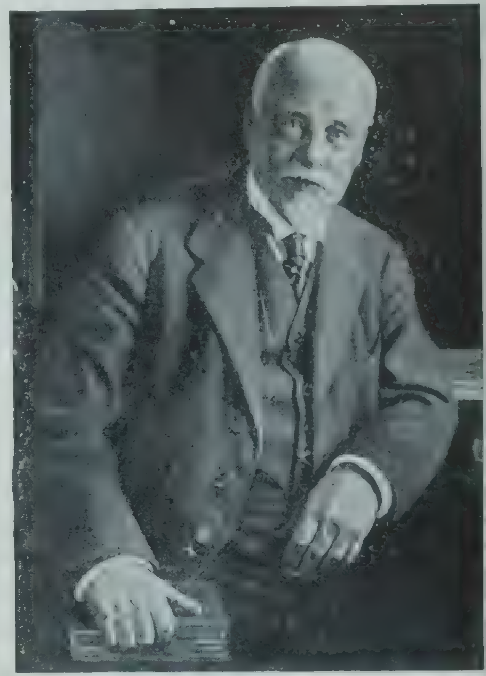

Fig. 381. William H. Welch. (From the painting by Martin Frobisher, Sr. Photograph by the courtesy of Dr. W. G. MacCallum.)

Since Reed and his co-workers had not found any animal which was susceptible to the disease, and which they could use for the experiment, human volunteers were asked to live in the compartments for days; to be imprisoned with lurking, flying death; to sleep in the beds; live in the clothes and eat from the dishes soiled by men who had died of yellow fever. It was not a pleasant invitation but there were numerous volunteers among the American troops! For days nothing happened. Then, one after another, men in the mosquito compartment became ill with yellow fever. Fortunately none of the men died. The men in the other compartment remained well! The secret was at last wrenched from nature! The mysterious messenger of death was at last revealed as a certain tropical mosquito (*Aedes aegypti*). This mosquito lives *only* in towns and cities; *not* in rural or jungle areas. Meanwhile Lazear, allowing himself to be bitten in a yellow fever hospital by an infected mosquito, lost his own life. Carlos Finlay's "ridiculous ideas" were fully verified.[15]

Although the *means* by which yellow fever was transmitted was known, the actual *cause* of the disease was not. Walter Reed, at the suggestion of that great physician and founder of medical science in America, William Henry Welch (Fig. 381), tested the filterability of the infectious agent in the blood of yellow fever patients and easily proved that the causative agent was not an ordinary bacterium but an *invisible, noncultivable, filter-passing* virus. Thus, not only was the transmitting insect but the nature of the causative agent revealed.[16] It is evident that the disease may attack susceptible populations in areas where *Aedes aegypti* are numerous. These are always cities since *A. aegypti does not breed in jungles*. Epidemics have occurred during summer months in many northern seaports, the mosquitoes and virus being carried from the tropics by ships. The danger of airplane-borne yellow fever is obvious, since many places have large numbers of *Aedes aegypti,* including India and our Gulf states.

The remainder of the story is one of confirmation of Reed's work, study of the virus, campaigns against *Aedes* mosquitoes and production of an effective vaccine. Yellow fever disappeared from Cuba! The Panama Canal became a practical project. The islands of the Caribbean became more healthy, and the "yellow jack" retreated, snarling, as it were, to the interior of South America. In 1932 Soper, Cardoso, Seraphim, Frobisher and Pinheiro made the discovery that yellow fever persists in remote parts of the wooded and rural portions of the continent, where it is transmitted by biting insects other than *Aedes aegypti* and is maintained among wild animals as well as human beings. It is referred to as "jungle yellow fever."[17, 18]

Two of the most important discoveries were the susceptibility of *rhesus* monkeys[19] and of white mice[20] to the disease. These findings greatly facilitated research, as no means of maintaining viruses in eggs or tissue cultures were then known and large scale human experiments were impracticable. Complement-fixing antibodies were first demonstrated in yellow fever by Frobisher[21, 22] and have since been found in many other viral diseases.

Immunization Against Yellow Fever. As previously noted, natural yellow fever virus affects chiefly the viscera (viscerotropic). When yellow fever virus, modified by mouse-brain passage so as to become *neurotropic,*[23] as previously described, is injected into the human body by any route *other than the nervous system,* no visible attack of yellow fever occurs but the virus stimulates the production of very potent antibodies and the person is thenceforth immune to yellow fever. However, the danger of invasion of the nervous system is an always-present danger.

A very curious phenomenon related to this variation in tropism was observed after it was found possible to cultivate the yellow fever virus in chick-embryo Tyrode-solution medium. Neurotropic virus completely lost its neurotropic *virulence* if the *nervous tissues* (brain and spinal cord) of the chick embryos were removed before mincing their tissues for the medium! The virulence of the virus was so reduced in such cultures that it seemed incapable of producing either the classical viscerotropic or the neurotropic form of the disease. It did, however, produce immunity, and it has been used to immunize millions of persons. It was later found that this loss of virulence could occur as a result of natural variation.[23, 24] It was also found that the virus multiplies greatly and rapidly in the embryonated chicken egg. The phenomenon has proven to be an excellent field for research in connection with other viruses.

The present method of preparation of yellow fever vaccine consists in inoculating a virus of reduced virulence (17D virus) into chick embryos in the shell. The inoculum for the embryos consists of fluid from a tissue culture of measured, attenuated virulence. (See section on methods of cultivation, page 149.) Further steps include incubating for about four days and then removing the embryos and grinding them very finely in a suitable suspending fluid. This vaccine is highly satisfactory and produces enduring immunity.[24] Certain initial difficulties with jaundice due to the inclusion of a certain icterogenic factor in human serum in the suspending fluid were overcome by discontinuing the use of the serum.[25] In French West Africa an interesting use of two living agents as vaccines simultaneously, yellow fever and vaccinia, in the same site has been reported to be successful. It has the advantage of simplicity but requires further study.[26]

The usual procedure consists in subcutaneous inoculation much as typhoid vaccine is given. The immunity afforded by the vaccination is high grade and durable like natural immunity.[26a]

POLIOMYELITIS

Infantile paralysis or, more properly, anterior poliomyelitis, is an acute febrile disease, world-wide in distribution, most common in children as a rule but not confined to childhood, and is caused by a virus. It is characterized, as are many viral diseases, by sudden onset and a febrile attack with nausea, headache, sometimes stiff neck, and muscular pains. There is evidence that in many instances the disease is short and mild, often passing unrecognized, with complete recovery and presumably life-long immunity. In a relatively small percentage of cases the disease is severe, and myelitis (muscular disease) with paralysis and degeneration occurs. There is extensive damage to nervous tissues, including those which are in association with the muscles, as well as to the walls of blood vessels.

The virus is well preserved by freezing at —76° C. and by lyophilization. It is probable that pasteurization inactivates it though traces of it may possibly survive that temperature.[27] It is also readily inactivated by ultraviolet irradiation,[28] and is probably inactivated in drinking water supplies by the usual processes of chlorination, though some data suggest that it may be necessary to increase the chlorine dosage.[29] Studies of the virus show it to be one of the smallest ($10–15\mu\mu$). It is possible that it may exist as liquid crystals somewhat like plant viruses.[30]

The virus appears to gain entry to the body through exposed nerves in mucosal surfaces of the upper respiratory tract, mouth, and intestinal tract.[31] Data have been advanced in support of the idea that extraction of teeth and throat operations such as tonsilectomy, may favor infection with the virus by exposure of nerves. The point appears debatable.[32] It multiplies in the nervous tissues[33, 34] and possibly other tissues.

The virus probably multiplies primarily and very commonly in the gastro-intestinal tract. Only when it then invades nervous tissues does the recognizable form of the disease occur. As in epidemic meningitis, there are probably a great many infections yet only occasionally does disease occur.

The virus leaves the body in the feces[35] and in oral and nasal secretions[36] of patients as well as of persons without any symptoms of disease. Thus the role of carriers in dissemination is *very important.* Apparently animals do not harbor the virus in nature, nor is there any evidence that biting insects transmit the disease.[37, 38, 39, 40] However, it has been shown that the serum of dogs may neutralize the virus of the Lansing strain of virus,* suggesting that these animals may be involved in spread of the disease.[41]

There are several strains, with somewhat different geographical distributions, clinical peculiarities and immunological differences. Laboratory investigation of poliomyelitis has been hampered by the fact that monkeys were for a long time the only known susceptible experimental animals. A strain of poliomyelitis virus now referred to as the Lansing or mouse-adapted strain was isolated in monkeys in 1937 by

* A virus modified by passage in mice, but derived from poliomyelitis virus.

Armstrong from a fatal human case. By passage of the virus through eastern cotton rats and later through white mice, it became adapted to mice so that it could be used for experimental purposes, such as tests of the neutralizing power of patients' and convalescents' sera. Only a few poliomyelitis viruses have undergone adaptation to mice.[42, 43, 44] A few have been adapted to embryonated eggs, an achievement pointing toward development of a vaccine.[45]

The distribution of poliomyelitis virus, as indicated by tests for neutralizing antibodies against the Lansing or one of several other strains, with sera collected among various populations, seems to be fairly general and widespread although only occasionally does a severe paralytic or fatal case occur. The means of transmission is not clear. The virus is found in the human intestinal tract[35, 46] and has repeatedly been found in feces of patients and of convalescent carriers and normal persons. It also occurs in sputum, sewage, and house flies[47] from privies containing infectious feces and therefore probably finds many pathways open for transfer from one person to another.

It is notable that, after feeding blow flies on feces from poliomyelitis patients, the virus could be obtained in the feces of the flies for as long as three weeks afterward, strongly suggesting that the virus multiplies in the flies.[48] This, with the occurrence of poliomyelitis epidemics usually during the "fly season" suggests the view that flies are an important vector of poliomyelitis. However, it is evident that flies are not alone responsible, as cases have occurred in areas where flies have been eliminated by D.D.T.[49] The epidemiology of the disease is more fully discussed by Maxcy[37] and by Sabin.[40] Cases seem to develop particularly during summer and fall, and more commonly in suburban and rural areas than in cities. There are no generally accepted means of prophylaxis. Complete avoidance of sputum, sewage and house flies might or might not give protection, provided it were possible so to isolate oneself.[39] No vaccine is generally available yet, but Morgan has been able to increase resistance in monkeys by means of virus inactivated with formaldehyde.[49a]

HUMAN INFLUENZA

This virus apparently enters and leaves the body via the oral and nasal tracts. The symptoms of influenza are often confused with those of the common cold. They may be present in cases of either colds or influenza. The most distinctive symptoms of a cold, however, are related, as a rule, largely to the nose and upper respiratory tract. Fever is mild; recovery rapid; fatality nil. Influenza on the other hand, is generally described as a markedly febrile disease, extremely weakening, accompanied by muscular pains, sweating, and sometimes by the symptoms of the common cold as well. Recovery is often delayed for weeks and fatality is sometimes high.

Types of Human Influenza. It is essential to differentiate between the several types of human influenza. First, there is *endemic* influenza. Clinically this is indistinguishable from the *epidemic* disease. Endemic diseases are those which are always present in relatively constant numbers in the population, cases or small groups of cases occurring sporadically here and there, now and then. It may be that cases of what are called endemic influenza represent several clinically similar but etiologically different diseases.

An important one may be Newcastle disease, formerly thought to be confined to poultry. Antibodies to this virus have been found in numerous human beings following influenza-like or "mild-polio"-like attacks.[39b]

Epidemic influenza, on the contrary, has a more distinct yet not unique etiology. At least two types of epidemic influenza virus have been discovered. The older one, now called influenza virus A, seems to be most widely distributed. Influenza virus B was differentiated in 1940 by Francis[50] and by Magill.[51] The A and B viruses, while causing clinically indistinguishable diseases, and although very much alike, are entirely distinct in an immunological sense. Other varieties of influenza virus, some quite distinct and some more or less closely related to A and B, have been described. These strain difference are of the greatest importance in respect to the preparation of an effective vaccine, because infection with some strains gives no protection whatever against certain others.[52, 53, 54]

The type of influenza which occurred in highly lethal form in the 1918–1919 world-wide epidemic may have been due to still another form of the virus, which is sometimes differentiated as "the virus of pandemic influenza." The true etiology of the 1918–1919 epidemic is still a matter of surmise.

Influenza Virus. Because of the enormous importance of influenza as a disabling disease, and one not infrequently fatal, especially in ill or infirm or weakened persons, it has been subjected to many studies. The virus is about $100\mu\mu$ in diameter. It is easily inactivated by heat (even by 37° C.),[55] ultraviolet irradiation, disinfectants,[56] and does not survive well in saline solution, but does in a solution buffered at pH 7. It may be cultivated readily in the allantoic fluid of 10- to 11-day-old chick embryos at about 36° C. It survives well at —75° C. Electronographs show it to be spherical. It is readily filterable. Attempts to separate the virus (*or any animal virus*) from animal matter have not been wholly successful.[57–59]

Apparently influenza virus is highly poisonous per se; *i.e.,* contains an endotoxin. Toxicity is apparently separable from infectivity, and may be neutralized by specific immune serum.[60]

Cultivation in Embryos. Early studies of influenza virus were made by inoculating ferrets intranasally with nasal washings. Later it was found that mice were susceptible to intranasal infection and that the virus could be then carried into chick embryos. In 1937 it was found possible to infect chick embryos directly and the method is now widely used. Nasal washings,.centrifuged to remove extraneous matter, are treated with penicillin and streptomycin to control bacterial infection and injected into the amniotic sac of the embryos. The phenomenon of chicken-red cell agglutination (described below) is used to detect the presence of virus in the egg fluids after forty-eight hours at 35° to 36° C. If there is doubt, the fluids are inoculated into new embryos and the process repeated four or five times until enough virus is present to agglutinate red cells or the test is declared negative. Such a process is often called "blind passaging." The details are summarized well by Salk.[61] The method is widely used in virus studies.

Agglutination of Erythrocytes. Two techniques to be described are extensively used in studies of influenza and other viruses.[62, 63] If washed chicken or human group O erythrocytes in about one per cent concentration in saline are mixed with virus-containing material such as infected embryonic chick fluid, and held for one to two hours at room temperature, the red cells are agglutinated. They appear to combine with the virus. By measuring carefully the amounts of virus-fluid, and red cell suspension, time of inoculation, method of reading the result, etc., accurate quantitative results may be obtained.

The degree of agglutination may be read by measuring decrease in turbidity of the cell suspension in a photoelectric turbidimeter (the Hirst method) or by noting the pattern of the sedimented cells on the bottom of the tube. A smooth, compact "button" with regular edges is negative; a definitely granular sediment with irregular edges indicates agglutination. This is called the "pattern" method of Salk. Extensive studies of factors affecting growth in embryos, agglutination and animal infectivity have been published.[64, 65]

That the combination of influenza virus with red cells is not a purely physical effect is seen in the facts that (1) after being *eluted* from (washed off of) erythrocytes the virus will not longer combine with other red cells; and (2) red cells from which virus has been eluted will not adsorb more virus. A substance has been obtained from erythrocytes which inhibits combination of the virus with the cells. This may be the "receptor" substance of the erythrocytes, with which the virus combines physico-chemically.[66]

Erythrocyte agglutination is not produced solely by influenza virus. The viruses of mumps, smallpox and Newcastle disease and some others have the same property. Further, substances other than viruses will produce red-cell agglutination. In 1949 the list included certain bacterial suspensions and botulinal toxin.[67]

Immunological Phenomena. In man and animals infected with influenza and several other viruses antibodies develop which may be demonstrated by (1) neutralization of the virus, using infection of mice as indicator; (2) complement fixation, using as antigen virus derived from egg embryonic fluids; (3) inhibition of erythrocyte agglutination.

In the first, dilutions of the serum to be tested are each mixed with a standard lethal dose of virus and instilled intranasally in mice. Survival of the mice indicates presence of antibody.

Methods of complement fixation have already been described (see page 319).

In the agglutination-inhibition test the serum is diluted serially and to 1 ml. of each dilution is added a standard suspension of red cells and virus. In the presence of antibodies, no agglutination occurs. Details of good diagnostic procedure are given in the references.[61, 63a] The practical use of some of these methods in the study of an influenza epidemic is well illustrated by Ward and Leymaster.[67] Modifications of these procedures are constantly being developed.

Influenza Vaccination. A very extensive literature is available on this subject, some of which is cited at the end of this chapter. Vaccines for several viruses other than influenza are made by processes based on the same principles. Here we may discuss methods of preparation or influenza vaccine and results obtained.

Vaccine Preparation. Several methods are used. The aim of all is to cultivate the virus in egg embryonic fluid, and then by various means, to (1) concentrate the virus and free it from egg proteins as much as possible; (2) inactivate it. For concentration one method is adsorption of the virus onto the red cells of the chick embryos in which the virus is grown. Blood vessels of the embryo are ruptured when removing the allantoic fluid. The virus adsorbs to the erythrocytes in two hours at 4° C. The cold fluid is then centrifuged. The supernatant fluid is discarded and the sedimented cells, with adsorbed virus, are resuspended in 1/10 the original volume, using saline solution. The suspension is then held at 37° C. for four hours, while the virus is eluted (leaves the red cells). After centrifugation the red cells are discarded, leaving the virus concentrated in the saline. The potency of the vaccine is measured by its infectivity in animals and by hemagglutination tests. It is then inactivated with formaldehyde and a disinfectant is added to prevent growth of bacteria. Virus A and B and other strains are pooled.[68] The immunogenic potency and sterility are tested in mice. Many difficulties were overcome in standardizing vaccines. Much of the work was done in conjunction with the U. S. Army.[69]

Stanley et al. found that they could produce a product of high purity and concentration by simple high-speed centrifugation. The embryonic fluids are harvested as free from red cells as possible. Formaldehyde and phenyl-mercuric-nitrate are added. The fluids are then spun at intermediate speeds to remove cells, debris, etc. The use of alcohol as a precipitant to aid contrifugation appears valuable.[70, 71] The fluid thus cleared is then ultracentrifuged (30.000 r.p.m.). The virus collects in the bowl of the centrifuge and the virus-free fluid passes on. The virus is then removed from the sides of the bowl with buffered saline solution, reclarified and bottled for use.

Results of Vaccination. Experience with influenza vaccination has varied. For example, the Army was encouraged by early results and adopted influenza vaccination in 1946.[72] The view generally held in 1949 was that vaccine containing A and B viruses gives a valuable degree of, but not complete, protection and is well worth while.[73, 74]

Unfavorable experience was reported in 1948 when an influenza A outbreak occurred in a military academy supposedly immunized by vaccine, and in a boys' school. The failures of vaccination were apparently due to strains of virus antigenically different from those used in vaccination.[75, 76]

MUMPS

This disease was so clearly described by Hippocrates as to leave no doubt that it has long afflicted the human race. It characteristically causes swellings of various salivary glands, notably the parotids under the ears, but also attacks testes and ovaries in sexually mature persons, as well as other tissues. Many persons apparently become infected and immune early in life without any perceptible disease or discomfort. Second attacks may occur. Mortality is low. Because of the destructive action of the virus on adult testes and ovaries it is desirable to become infected and immune before puberty.

The virus is cultivable in chick embryos, and eggs have served for direct isolation of the virus from patients.[77] The virus of the eggs can be used as antigen in complement fixation tests to establish a retrospective diagnosis. Like the influenza virus, mumps virus after egg passage causes hemagglutination which can be inhibited by immune serum. The embryonated egg also serves well as a test animal in measuring the antibody content of serum of persons convalescent from mumps.[78]

Passive Protection. Serum, or serum globulins, taken from persons recently recovered from mumps offer a means of temporary protection, at least from severe damage and possible sterility in adults if given early in the course of the disease.

Vaccine. By passaging the virus for long periods in chick embryos it appears to lose virulence (for monkeys) but produces a solid immunity. Data on its value for human beings are being collected.[79]

HOMOLOGOUS SERUM JAUNDICE

This is a disease of the liver causing jaundice. It occurs about six weeks to six months or more following injection of human blood or serum or plasma, containing the virus. The virus often is present unrecognized, *i.e.,* it seems to cause inapparent infections, or exists in carriers. It resists freezing and drying. It was this virus which caused difficulty following the injection of yellow fever vaccine early in the recent war, and the yellow fever vaccine episode led to the discovery of the virus.[80] Apparently gamma globulin as used in preventing measles, etc., does not transmit the disease.[81] The virus is presumably a typical filterable one and is quite resistant to drying, climatic temperatures, etc. It can be inactivated by ultraviolet irradiation.[82] However, it has been impossible to gain much information about it because man is the only known experimental animal. Further data on control, cases, etc., are to be found in the literature.[83, 84, 85]

INFECTIOUS HEPATITIS

This disease is clinically indistinguishable from homologous serum jaundice and the two may be closely related. However, the homologous serum agent appears to be transmitted only by human blood or serum. The virus of infectious hepatitis seems to occur in serum, feces and urine and hence in sewage-polluted water,[86] in milk,[87] etc., and is probably also transmitted by close contact, in saliva, etc. It may possibly be transmitted by flies and biting insects; also in the same manner as homologous serum jaundice. It appears to be rather resistant to chlorine, as used in disinfecting water, and 56° C. for at least 30 minutes. Immunity seems to persist for at least six months after recovery.[88] Mortality is low.[89] Further data may be found in the literature cited.[90, 91, 92]

MEASLES

Measles is so common that little description is needed though a clinical diagnosis is sometimes not easy. It is believed that susceptibility of man is universal, except in infants of immune mothers up to six months of age. Natural active immunity is high grade and lifelong.

The disease is entirely distinct from German measles. The early symptoms are much like those of a cold. The oral and nasal secretions at this time are highly infective. Measles during this stage is probably the most readily transmitted of all infectious diseases.

The virus is cultivable on the chorio-allantoic membrane of chick embryos. After several egg passages the virus appears to lose virulence and produce a very mild infection in children. Thus there is a possibility of producing a vaccine.[93]

Temporary passive protection by means of antibodies (gamma globulin) in the serum of immune persons is used to prevent the development of measles in children under about five years of age since in them the disease is most likely to be followed by pneumonia and other dangerous complications frequently fatal. In older children the dosage may be reduced so that a mild immunizing attack is permitted to occur in childhood. This prevents a possibly costly and certainly inconvenient lay-up during adult life.

COMMON COLD

In 1947 Pollard and Caplovitz established the virus of the common cold in embryonated eggs. No evidence was obtained by hemagglutination and other tests that an influenza virus was involved. Egg passage appeared to attenuate the virus. Subcutaneous injection of egg-grown virus appeared to confer immunity to intranasal instillation of virulent virus from colds in human beings. Not all persons who recovered from intranasal infection were immune to later experimental infections.[94, 95]

Topping and Atlas also produced common colds in human volunteers with unfiltered nasal washings of patients, and established the virus in embryonated eggs. With the allantoic fluid, etc., of these they produced the diseases in human volunteers. No elementary bodies were seen in the infectious materials. The virus is apparently not infectious for mice as is influenza virus, nor for rats, rabbits or some other animals. Further, recovered persons showed no rise in antibody titer against influenza viruses A or B. The cold virus survived several weeks at —50° C. Electronographs revealed characteristic particles different from influenza particles.[96]

The importance of these studies, especially the possibility of artificial immunization is obvious in view of the estimated loss of 80,000,000 man-days of work annually due to common colds and the expenditure of $4,000,000 by the public annually on remedies which do not cure colds.[97]

PRENATAL INJURY BY INFECTIOUS AGENTS

It has long been known that syphilis may greatly injure and often kill the unborn child. Other bacterial infections, especially typhoid fever, occurring in pregnant women are also known to affect the child unfavorably. Among the viral diseases influenza was seen to have a disastrous effect on pregnant women and unborn children in the great pandemic in 1918. About half of the expectant mothers died. Of the survivors about 60 per cent had miscarriages (abortions) or lost the children at birth or soon after. In 1941 Gregg in Australia noted that children born of mothers who contracted rubella (German measles) during *the first three months* of pregnancy frequently had defects of hearing and eyesight or deformities of the heart, feeblemindedness, and other more striking defects. Confirmatory data were collected elsewhere, including the United States.[98]

German measles, previously generally regarded as of little consequence, is presumably caused by a virus which has a predeliction for *early embryonic* tissues upon which it exerts a destructive effect; hence the various malformations mentioned above.

The importance of the problem of prenatal injury by various infectious agents was brought into sharp focus by these observations. It may be that Shakespeare realized some such relationship when he wrote: "That foul fiend Flibbertigibbit—gives the web and pin (*i.e.*, cataract), squints the eye, and makes the harelip." Flibbertigibitt may be rubella or other infection of expectant mothers early in pregnancy.

Because no vaccine is available, some pediatricians recommend exposure of girls to the disease between the ages of 6 and 8 years so that they may become immune and thus do not contract the disease during pregnancy later in life. In 1948 the suggestion was made that non-immune mothers could be protected during early pregnancy by passive immunization with an antibody-bearing fraction (gamma-globulin) of the blood of immune persons.[99]

RABIES

This disease has been discussed in the chapter on immunity since Pasteur's studies on rabies vaccination are of historical immunological interest. The virus occurs in the saliva of infected animals and is transmitted by their bite. It attacks the central nervous system and produces characteristic cytoplasmic inclusions called Negri bodies. By examination of stained smears or sections of the brain of rabid animals these can often be seen, and used as the basis for diagnosis. It is now thought that they are not entirely specific to rabies. A more reliable but more time-consuming means of diagnosis is to inject saliva of rabid animals or persons, or the suspect brain tissues, into the brain of normal mice, guinea pigs or other animals. These will develop the typical disease.[100] Immune serum can be used to protect the test animals, thus confirming the diagnosis. Such serum is not known to be of value in human therapy. Complement fixation tests can also be used in the study of the disease.[101]

The disease is primarily one of animals, occurring in all canines and in animals which they bite, including domestic stock, cats, and many wild animals, which transmit it to each other by fighting, predatory habits, etc. All appear to succumb to the infection.[102, 103]

A dog which bites a person should be confined and kept under observation for at least ten days. If rabid, it will die after exhibiting definite symptoms which include irritability and restlessness, anxiety, fear, and failure to recognize its master; followed by paralysis of the throat and inability to eat or drink, drooling of saliva, and finally general paralysis and death. If not confined it has a tendency to wander long distances and to bite various objects, animals and persons (furious rabies) unless paralysis sets in earlier when it may merely skulk or run about dumbly (dumb rabies).[104] In cats the furious stage is terrifying to observe and the animal is exceedingly dangerous because he covers himself with saliva and the slightest scratch of tooth or claw can infect. He will attack on sight. Even objects wetted with the saliva of a rabid animal can infect.[100]

The bitten person should consult a physician immediately. Immediate cauterization of fresh bites with fuming nitric acid is advised. Pasteur treatment should be instituted at once. If the animal is under observation and shows no symptoms after ten days, the treatment may be discontinued. Some authorities delay treatment during the observation period, as the series of injections is unpleasant at best. If the animal is not caught, the treatment should be completed.

The control of rabies (since no cure is known) consists in vaccination and licensing of dogs and cats, destruction of strays, quarantine when the disease is prevalent, etc., and general measures to prevent spread among animals.[104, 105]

Rabies is not transmitted exclusively by quadrupeds. This was revealed when Johnson found that derriengue, a highly fatal paralytic disease of cattle in Mexico, is in reality rabies and is transmitted among them by vampire bats, who maintain it among themselves by fighting, etc.[106]

THE PSITTACOSIS-LYMPHOGRANULOMA GROUP

This group of viruses includes, besides the agents of psittacosis and lymphogranuloma venereum, the viruses of vaccinia and smallpox, fowlpox, inclusion conjunctivitis, meningoencephalitis, feline pneumonitis and several others. These viruses differ markedly from such minute agents as yellow fever, influenza and poliomyelitis virus, especially in size (see diagram, page 796). They approach the smaller bacteria and the rickettsiae in dimensions. The elementary bodies are spherical and range from about 200 to 300$\mu\mu$ in diameter. They can be stained with aniline dyes (gram-negative) and studied with ordinary microscopes. They are further distinguished by their rather complex life cycle which is strongly suggestive of the organisms of the pleuropneumonia group discussed in the next chapter. However, unlike the pleuropneumonia organisms, which grow in lifeless media, none of the psittacosis type of viruses has been cultivated in the absence of living cells. Many of these viruses undergo cyclical morphological changes as they develop in the cells of infected animals.[107] These morphological changes in lymphogranuloma virus, a representative member of the group, involve multiplication of the elementary bodies or initial bodies into chains and masses (plaques), the masses finally enclosing themselves in little vacuoles or vesicles in the infected cells and tissues. The vesicles and masses of granules increase in size and finally rupture, liberating many new-formed elementary bodies in the surrounding tissues. Similar structures and cyclical changes are seen in the other viruses in the lymphogranuloma-psittacosis group, but whether they represent the same processes and have the same significance in all is not clear. Electronographs of the virus of fowlpox and of feline pneumonitis have helped clarify this question somewhat.[108–111]

A significant observation in regard to the lymphogranuloma and related pathogenic agents is that they form toxins similar to bacterial endotoxins against which specific antitoxic sera may be produced.[112]

Most of the viruses of this group are cultivable in chick embryos, multiplying especially well in the yolk sac.[113] They may also be developed in the brains of experimentally injected mice or in the pus from draining buboes. This makes it possible to obtain large amounts of the elementary bodies for use in research and diagnostic tests.

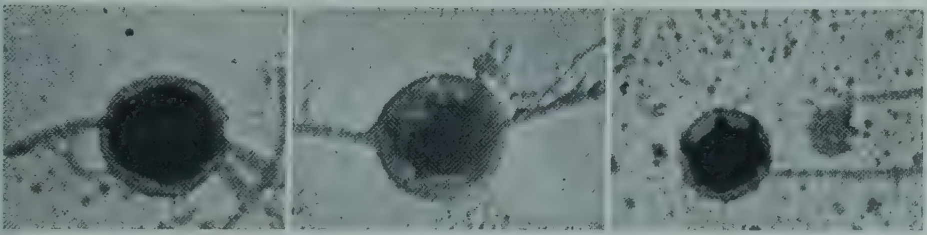

Fig. 382. Electronograph of bodies thought to be the elementary bodies of psittacosis. Various forms are seen. These were cultivated in a living chick embryo. The fibers are from the egg itself. (F. Heinments and O. J. Golub, J. Bact., vol. 56, 1948.)

Psittacosis (Parrot Fever). Psittacosis virus is representative of the group. It illustrates several points in common, and also in contrast, with such viruses as that of poliomyelitis and yellow fever, and it will be of interest to discuss some of them briefly. The fact that human beings can contract a form of pneumonia from sick parrots was recognized by Ritter as early as 1880. The disease is a dangerous one. Various bacteria were designated as the cause of the disease, but as in yellow fever, it was found that the causative agent is not a bacterium; is present in the blood (and sputum) of human patients as well as in tissues of man and birds; and will pass through Berkefeld filters.[114]

Large elementary bodies are found in and about tissue cells of organs (spleens, livers) of infected parrots, mice, etc. These appear to have a complex structure and may contain more than one antigenic substance. They are often called LCL bodies, recalling the names of their discoverers: Levinthal, Coles and Lillie. They differ from all other members of this group in being stainable by both Feulgen and Castanedas methods* (Fig. 382).

This virus, like other viruses, will not grow except in contact with living tissue cells. It may be cultivated in tissue cultures and chick embryos. On the chorio-allantoic membrane pock-like lesions are described. Thus we see that the virus of psittacosis partakes of some of the properties of both yellow fever and smallpox viruses, but shows differences from each which illustrate the danger of making generalized statements about any virus.

Psittacosis is *transmitted* by dried nasal secretions and feces of parrots and such infected material may be blown or otherwise transmitted for considerable distances from the infected birds.[116]

In man, if the disease is contracted naturally, *i.e.*, by inhalation of dust infected by birds, the disease takes the form of a pneumonia, the virus being demonstrable in the sputum and blood. This is used for diagnosis by injection into mice. Transmission from man to man is uncommon.

Immunity. Man and animals, after recovery, are somewhat refractory to reinfection, but immunity to psittacosis is not so marked, prolonged or readily demonstrable as in yellow fever or smallpox. The presence of immune bodies in the blood of animals or men recovered from the disease is demonstrated by mixing their serum with infectious material (ground-up spleen, etc.), and injecting it into mice. The mice are, in great part, protected. This is called a *neutralization test*. Similar tests are used in the demonstration of yellow fever antibodies, as well as antibodies against poliomyelitis and a number of other viral diseases.

Abnormal Portal of Entry. Psittacosis virus, injected into monkeys or human beings *intramuscularly* (as contrasted with intracranial and respiratory routes of infection), produces only a mild illness which results in a relatively refractory state. This fact has been made use of in the protection of human beings engaged in laboratory investigations of the disease. It is evident that the portal of entry plays an all-important role in psittacosis as well as in yellow fever. It is important to note that *intraperitoneal* injection of vaccine will immunize to challenge doses given by the *same route*, but only about one half as well against *intranasal* challenge doses, even though agglutinins and other antibodies may be demonstrable in the blood.

Ornithosis. Psittacosis, or a disease much like it, occurs in many birds,[117] including canaries, pigeons, etc. In such birds the disease is called ornithosis. Many species of seashore birds have been shown to contain the virus, or immune bodies to the virus,[118] which is distinguishable from that of true psittacosis only with difficulty.[119]

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* The virus of lymphogranuloma venereum stains only by the Castaneda method; vaccinia by neither.[115]

Pigeons often become infective carriers. Of 100 wild pigeons caught in Baltimore, Md., fifteen were found infected. However, experience shows that human disease rarely results from the presence of ornithosis in the birds under ordinary circumstances.[120] English sparrows may be experimentally infected but have not been found naturally diseased.[121]

Lymphogranuloma Venereum. This disease, probably world-wide in distribution, though more common in tropical regions and in the Negro race, is due to a virus having many properties in common with that of psittacosis. The disease is transmitted chiefly by sexual contact, rarely by other means, and is most prevalent in sexually promiscuous persons during the ages of greatest sexual activity. The virus has its portal of entry through various mucous membranes, especially those of the genitalia and anorectal region. It may pass through the skin, via abrasions and cuts, or through the respiratory tract. It is usually not so fatal as syphilis and often heals spontaneously. However, it is capable of doing great damage.

One diagnostic test consists in the intradermal injection of heat- or phenol-killed suspension of the elementary bodies. In persons who have been infected for about two weeks or more there develops, after forty-eight to seventy-two hours, a characteristic papule with swelling and redness around the site of the injection. This is known as a Frei reaction, having been discovered by Frei in 1925. Complement fixation tests with egg-yolk antigen are of value in studying the incidence and distribution of lymphogranuloma. No method of active immunization is available at present. Control at present centers around finding and treating cases and preventing the spread of the disease to others through sexual or other personal contact.

BACTERIAL INFECTIONS IN VIRAL DISEASES

Animals or persons infected with some viruses seem to be especially susceptible to simultaneous infection with certain bacteria. This secondary infection often produces severe or fatal results in virus diseases which, in the absence of the bacteria, are in themselves relatively mild. Colds or influenza, for example, may make the tissues unable to resist infection with pneumococci or streptococci. Good examples of this combined action are seen in hog cholera and, as shown by Shope,[11] in swine influenza (see page 789).

NEOPLASTIC DISEASES

One of the great mysteries of human pathology is the etiology and nature of "cancer." There is a great variety of cancers or, more properly, tumors (swellings) or neoplasms (*neo* = new; *plasm* = tissue) of both plants and animals, and there seems to be a variety of causative agents. Whether any of these is a living organism is still unknown with regard to human neoplasms, but several animal tumors are definitely known to be due to infections with viruses, while some plant tumors are due to bacteria (see section on plant diseases, page 710) as well as to viruses. Whether, by studies of the animal or plant tumors, the secret of human neoplasms may eventually be solved, no one can say. There are many suggestive analogies between the infectious animal tumors and human neoplasms, but the actual relationship, if any, is still obscure.

Neoplasms, Viruses and Mutation. The development of neoplasms in human beings has long been associated with mechanical irritation or injury. In the presence of irritation and injury, cells are killed and new growth tends to replace them. In the continued presence of the injurious agent it is readily conceivable that the cells of the new growth may be altered so that they grow awry and become malignant, independently growing cells.

More recently, x-rays, ultraviolet and other irradiations, certain coal tar derivatives (methyl cholanthrene, etc.), mustard oils, certain aromatic amines and other irritating and injurious agents have been shown to induce neoplasms. How these act on tissue cells is no more certain than the manner of action of other injurious and irritating agents but *one striking property of all of them is the power to produce genetically stable changes (mutations) in various cells,* bacteria, yeasts, flies, tissues, etc. In lower animals viruses also can induce neoplasms. Whether the viruses act to produce cell mutations is not known. Viruses have not been demonstrated in human neoplasms, unless we include, as neoplasms, warts, laryngeal papillomata and condylomata acuminata (venereal warts).

Neoplasms thus appear to represent mutated cells which, because of some genetic change become, in essence, foreign to the tissues and body of origin, no longer are held in check by the normal growth regulating hormones, etc., and grow independently, sometimes slowly and within definite bounds (benign tumors), often without regard for normal limitations, spreading and invading everywhere, each tumor cell a malignant parasite. The connection between human neoplasms, viruses and genetically active (irritating, etc.) agents has not been finally established but as evidence accumulates it all seems to point in the same direction, i.e., that neoplasm cells are mutant cells,[122-125] and that viruses may produce some of these mutations.

Mutability of Virus. We have already seen that many viruses undergo alterations of virulence and tissue and species affinity by contact with new host tissues. We have also seen how bacteria undergo genetically stable change in contact with certain nucleic acids (pneumococci, see page 178). One of the most striking changes to be recorded is not in host or tissues specificity but from one virus to another. The mutagenic agent is an inanimate substance derived from one virus and applied to the other; the change being suggestive of the phenomenon observed in pneumococci or referred to on page 178 as entrainement. This change is seen when fibroma virus of rabbits "turns into" myxoma virus of rabbits. *Myxoma* is an almost invariably and rapidly fatal disease of *domestic* rabbits. The virus causes malignant, cancer-like, slimy tumors that occur all over the body.

Fibroma virus causes large, hard, warty tumors in wild cottontails (Fig. 383). These tumors have a closer superficial resemblance to benign human tumors (warts, which grow slowly and show little tendency to spread) than they do to malignant human cancer or infectious myxoma of rabbits. Fibroma disease is not usually fatal. Fibroma virus will acquire all of the properties of the myxoma virus when treated with heat-inactivated myxoma material.[126, 127]

Viral Diseases of Plants. In spite of the fact that our knowledge of viruses began with the discovery of a plant virus (tobacco mosaic) in 1892, only since 1920 have virus diseases of plants been extensively studied. They are of enormous agricultural and economic importance.[128, 129]

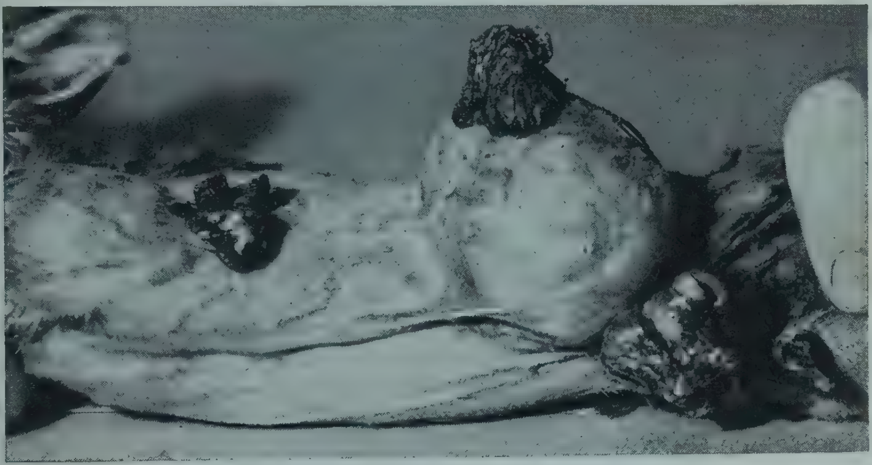

Fig. 383. Cottontail rabbit with naturally occurring virus papillomas. One has extended down and formed a subcutaneous mass. ("Virus Diseases" by Members of the Rockefeller Institute for Medical Research, Cornell University Press.)

A great variety of agricultural and other plants is known to be affected by viruses. The diseases caused are commonly spoken of according to the type of symptoms or changes induced in the plants. These changes may be classified under five main heads. (1) mottling and chlorosis; (2) distortion; (3) dwarfing and attenuation; (4) overgrowth; (5) necrosis.

Mottling is due to disturbances in cell metabolism which result in differences in intensity of the color of petals or leaves, and often extends to fruits. This is characteristic of the mosaic diseases of various plants (tobacco, potato, tomato). The lighter areas are said to be chlorotic (Figs. 384 and 385, *A*). "Yellows" of peaches is a good example of chlorosis. Irregular coloration of flowers, often very beautiful in effect, and seen in tulips ("color break") especially is a symptom of virus disease. The possible close relation between what is called "variegation" and virus disease has been discussed on page 805.

Distortion, consisting of wrinkling, puckering, or cupping of the leaves, often accompanies virus disease. Distortion may also result in a rolling up or curling of the leaves. Mosaic diseases such as rugose mosaic of potatoes, "curly-top" of sugar beets (Fig. 385, *B*) and cucumber mosaic show these symptoms very strikingly.

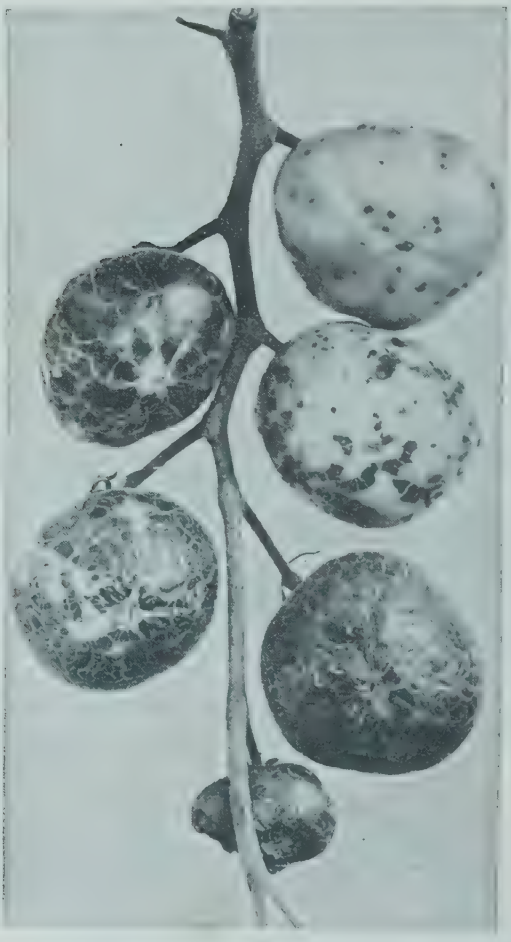

Fig. 384. Symptoms on tomato fruits produced by infection with a plant virus. (Photo by McKay, from Owens, "Principles of Plant Pathology," John Wiley & Sons, Inc., publishers.)

Dwarfing or attenuation is found in "witches broom" of potato and the mosaic of tomatoes (Fig. 386).

Overgrowth. Tumor-like growths or "galls" are often caused by bacteria (see page 710), but also sometimes accompany infections by viruses. For example, tumor formation is seen in "winter blight" of potatoes and in the "Fiji disease" of sugar cane. In the latter, the galls occur in the stalks and leaves.

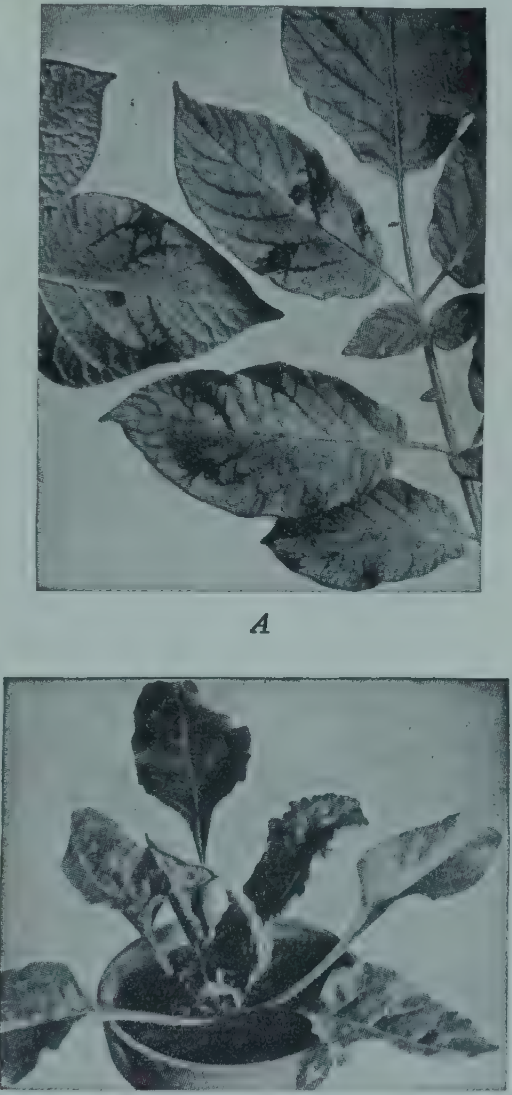

Fig. 385. *A*, Symptoms of rugose mosaic. *B*, Curly-top of sugar beet. (Photographs by McKay, Oreg. Agr. Exp. Sta., reprinted by permission from "Principles of Plant Pathology," by C. E. Owens, John Wiley & Sons, Inc., publishers.)

Necrosis, or death of the parts affected, may occur in any of these diseases. Bacterial decay soon follows.

A "blight" of citrus fruits, due to a virus, has recently been described and threatens citrus fruit growers with great economic loss and all of us with an increase in the price of oranges, etc.

Transmission of plant viruses is accomplished much as is transmission of the bacterial diseases of plants. The viruses usually have to be introduced through the outer layers of cells, as by pricking or abrasions. Transmission by leaf-eating or leaf-piercing insects is therefore especially important in natural spread of these diseases. A good example of the methods used in determining the insect vector of plant viruses is seen in a study of a disease of elm trees transmitted by leaf hoppers.[129a]

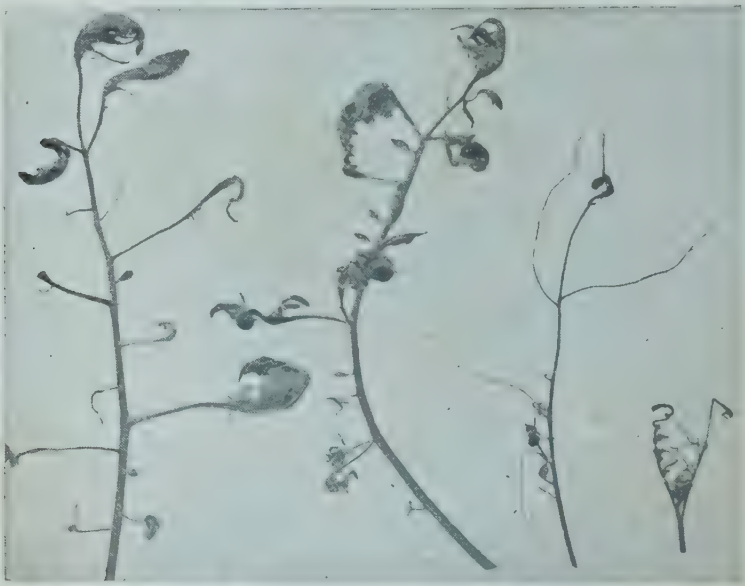

Fig. 386. Tomato leaves showing extreme attenuation due to infection with a plant virus. (Photo by McKay from Owens, "Principles of Plant Pathology," John Wiley & Sons, Inc., publishers.)

The same methods of control apply to both types (viral and bacterial) of plant disease. Actual contact between diseased and healthy plants is to be avoided; biting insects are to be controlled; instruments and gloves are to be disinfected, and weeds are to be eradicated. Rotation of crops, use of clean or disinfected seed and avoidance of infected new stock or tubers are other means of control.

Sometimes, as is true in bacterial diseases, individual plants will show resistance to a viral disease and recover. These are able to confer immunity upon susceptible plants of the same species when the latter are grafted upon the immune stock (Fig. 387).

Color Test for Plant Viruses. What may be a phenomenon of the most basic importance in plant, and possibly animal, virology is seen

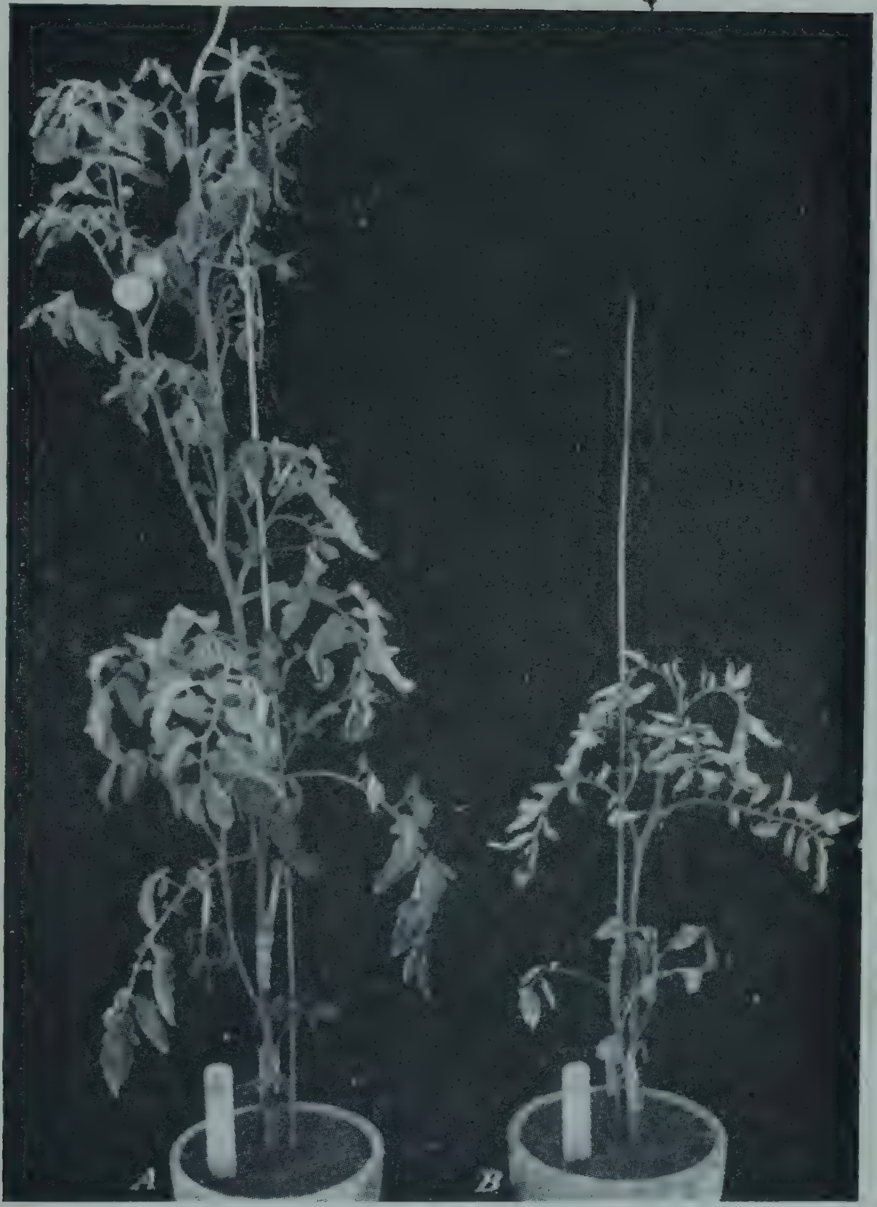

Fig. 387. Influence of protective substances on infection of tomato plants with curly-top virus, strain 3. *A*, Plant infected after grafting with a tobacco plant that had recovered following inoculation with virus strain 3. *B*, Non-resistant plant infected with the same virus strain by means of a beet leaf hopper inoculation. Photographed after 64 days. (Wallace, James M., in J. Agricultural Research, vol. 69.)

in the development of a brilliant red color by extracts of the leaves of peach or cherry leaves infected with certain viruses when treated in a particular manner. A disc of leaf, carefully selected as to kind of leaf, position in the leaf, etc., is boiled in aqueous solution of 4 per cent NaOH, 0.05 per cent $CuSO_4$, and 0.3 per cent Na citrate for five to ten minutes. The color develops in about ten minutes and is read in a photoelectric colorimeter. Normal leaves yield a greenish color; infected leaves, various shades of red. This test may have a practical value in locating viruses in imperceptibly infected plants and in studies of virus composition and host-virus interaction.[130]

FILTERABLE FORMS OF BACTERIA

Some workers are of the opinion that minute forms (gonidia, etc.), develop during special reproductive processes of bacteria and that these are so tiny that, when released from the cell, they can pass through filters as do true viruses. In this condition they are described as resembling, or actually belonging to, the group of *viruses*. Such forms are said by some to develop inside the bacterial cell wall by division of the cell contents without participation of the cell wall. This theory strongly suggests the life cycle of the pleuropneumonia-like organisms discussed in Chapter 50. It has been said, but never confirmed, that certain cultural conditions will induce the formation of virus-like forms by bacteria. The action of bacteriophage (see page 844) is also said to produce virus-like forms.

These possibilities cannot be denied but really convincing proof is nonexistent. On the other hand, a number of experimental studies make it appear highly improbable that bacteria are related to the viruses.[131, 132] Bacteria do not develop in viral diseases or in cultures of virus, nor viruses in cultures of bacteria as one would expect if the viruses were merely stages in the life cycle of bacteria. Many sources of error, which doubtless have misled some investigators, lie in the very complex problem of the physical properties of filters already referred to. It is evident that occasionally, even under the most carefully controlled conditions, when subjecting a culture of bacteria to the action of one of these filters, a bacterium may occasionally fail to be adsorbed and so appear in the filtrate and later multiply there, giving rise to the impression that some special type of filter-passing granule was present in the original culture. The growth may equally well have resulted from the passage through the filter of an abnormally small cell, perhaps a young cell or dwarf variant but these can hardly be regarded as virus-like. It also may have resulted from accidental contamination of the filtrate or a minute defect in the filter, eventualities difficult to avoid.

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Chapter 48

THE VIRUSES

III. BACTERIOPHAGE

One of the most interesting of the viruses is a parasite of bacteria. It was first described by Twort and later by d'Herelle and its action is often called the Twort-d'Herelle phenomenon. It is called *bacteriophage* (*bacterio* = bacterium; *phage* = eater).[1] Bacteriophage may be found in many situations in nature where bacteria are growing and is especially abundant in the intestine of man, animals and insects.

If a small amount of sewage, feces or ground-up flies or roaches be emulsified with broth and passed through a filter, the filtrate will often be found to contain this virus. It may be demonstrated by adding a drop or two of the filtrate to a very young, actively growing, slightly turbid, broth culture of appropriate bacteria. Virulent dysentery or typhoid bacilli often serve.

The culture will be found, after a few hours' incubation, to have become nearly or entirely clear and the live bacilli in it either reduced in numbers or entirely absent. If, now, this broth be filtered and a drop of the bacterium-free filtrate added to another young broth culture, this in turn will clear, and filtrates from it will induce the clearing phenomenon in other cultures. The lytic potency of the filtrates increases with each transfer, the bacteriophage multiplying enormously at each transfer and accomplishing the clearing much more rapidly and completely than at first. In addition to the increased activity of the 'phage, it usually becomes more and more specific for the bacterium on which it grows, *i.e.*, less active on other species.

If a loopful of the partly lysed (cleared) broth culture be smeared on an agar plate, any surviving bacteria that grow into colonies are often found to be distinct variants from the original, and it has been postulated that new species may originate in this way. The phenomenon is, indeed, very striking and offers interesting material for study. Smooth to rough variation is often induced in this way.

Plaque Formation. Multiplication of bacteriophage may also be made evident through an interesting phenomenon which may be produced by smearing an agar plate with a drop of the young, active culture *before* putting in the bacteriophage, and then smearing another agar plate with the same culture a few minutes *after* the addition of the bacteriophage. The first plate will show profuse, normal growth, smooth and gray, unbroken except in the most thinly seeded areas where there are separate colonies. These will have smooth and regular edges. The second plate will show less growth and it will be dotted here and there with "pinholes" or places where no visible growth has occurred. These "pinholes" are called bacteriophage *plaques*. Each may be regarded as a "colony" consisting of billions of particles of bacteriophage, which has destroyed the bacteria around it. Isolated bacterial colonies on this plate may show crescentic irregularities in their margins, as though pieces had been eaten out of the edges. They have been described as "mouth-eaten" colonies (Fig. 388).

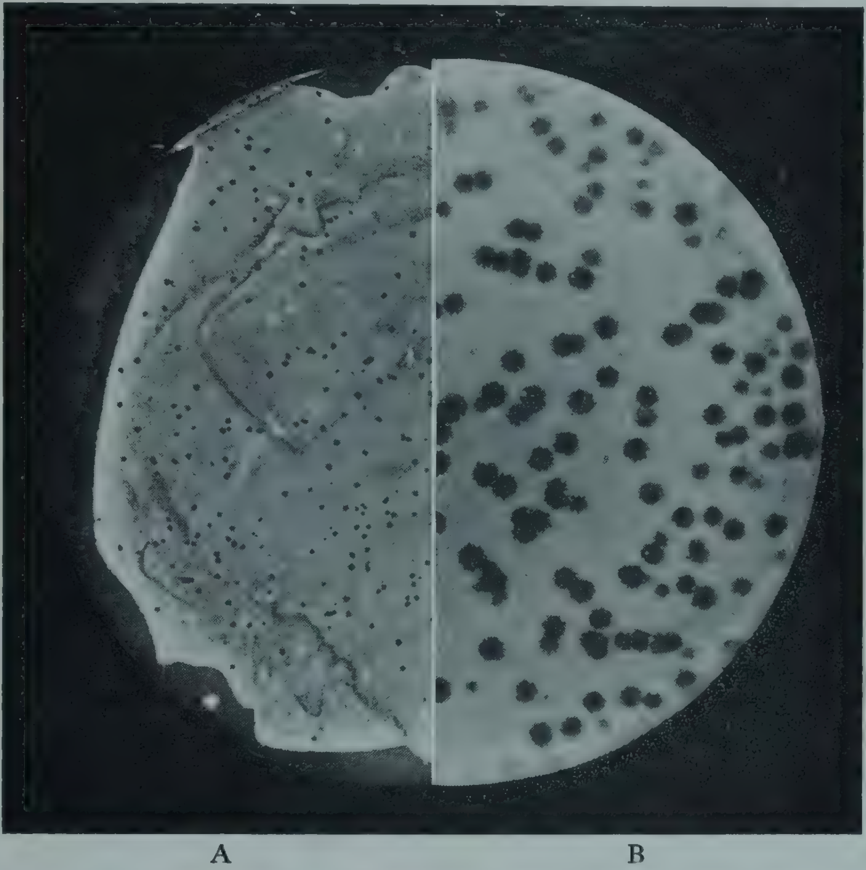
A B

Fig. 388. *A*, Plaque formation by bacteriophage. The dark "holes" are plaques. This is a "small plaque" variety of 'phage. (From unpublished data; Courtesy of Dr. Harry E. Morton and Elsie Laukford.) *B*, A large plaque variety of 'phage. Note the irregular edges of the plaques. The explanation of this is not clear. Minute colonies of resistant bacteria are often found in the area of the plaque. (Dr. J. Kleczkowska, J. Bact., 1945.)

Enumeration of Bacteriophage. This may be done by two methods: one involves the addition of graded quantities of the 'phage suspension being measured to constant amounts (say 5 ml.) of young broth cultures of the susceptible bacterium and, after appropriate incubation, noting either (*a*) the highest dilution of 'phage suspension producing complete or perceptible clearing in the cultures or (*b*) the time required for a standard decrease in turbidity of the culture to occur with a given dilution of 'phage.

The second method depends on counting the number of plaques produced on the surface of plates, inoculated as described above, by graded dilutions of the 'phage suspension.

The former method yields data on "potency" but rather inaccurate ideas of numbers of 'phage particles. The plaque method suffers from inaccuracies due to "clumps" of 'phage and to mechanical difficulties in spreading the 'phage suspension evenly over the solid medium. Much of this difficulty has been overcome by Yen who mixes 'phage and culture together with melted agar and pours the mixture into a Petri dish. After incubation the 'phage plaques are evenly distributed in the agar and on the surface. The usefulness of the method has been demonstrated by Kleczkowska.[2]

Multiplication of Bacteriophage. Like other viruses, bacteriophage will not multiply readily in any medium except living, bacterial cells. Dead cells, or dormant or old cells that are not *actively multiplying*, will not serve, although some experimenters seem to have obtained growth in dead cells or cell-free cultures under some conditions. Preferably young bacterial cultures are used. Bacteriophage will not *grow* in filtrates made from cultures which have been destroyed by the agent but will remain active in them for fairly long periods if refrigerated.

There have been many studies of the interaction between bacteriophages and their bacterial hosts in the hope of shedding light on the general problem of chemical and physical reactions which result in cell injury and virus multiplication. It has been shown that the addition of active or partly inactivated 'phage to suspensions of *Escherichia coli* in a synthetic medium causes an immediate cessation of multiplication of the bacteria.

In another investigation it was shown that 5-methyl tryptophane inhibits multiplication of *E. coli*. It does not kill the organism for many hours, nor does it kill bacteriophage. Yet in the presence of *host cell and 'phage* it prevents multiplication of the 'phage. This suggests the effect of malachite green and malnutrition on certain animal viral diseases in which, by disturbance of the metabolism of the host cell, multiplication of virus is suppressed (see page 801).[6]

A very curious phenomenon has been described by Maurer and Wooley in which pectin from citrus fruits, when added to cultures of *E. coli*, protected them from lysis and permitted them to grow in the presence of *active* 'phage which also *multiplied* but did not appear to cause lysis.[6a]

Structure of Bacteriophage. As shown by the electron microscope most bacteriophages appear to have a distinct "head" which may be round, ovoid or roughly cuboidal, and a "tail." Their general appearance is much like that of a young tadpole or a spermatozoan.

Action of Bacteriophage. Just how the bacteriophage acts upon the bacteria is not known. The dissolution of the bacteria seems to be entirely physical. At least, as shown by Ionesco-Mihaiesti there is no hydrolysis of the cell protein as by an enzyme.[3] It is thought by most, following d'Herelle, that the bacteriophage particles penetrate into the bacterial cells and multiply there, causing the cell to burst and liberate increased numbers of bacteriophage bodies (Fig. 392). The exact mechanism of bacteriophage action is not settled. Electronographs seem to support d'Herelle's views.[4, 5, 7, 10, 11] In any case the bacteria are actually destroyed in very short order, and the potency of the bacteriophage and the number of plaques producible with a drop of filtrate is greatly increased after lysis.

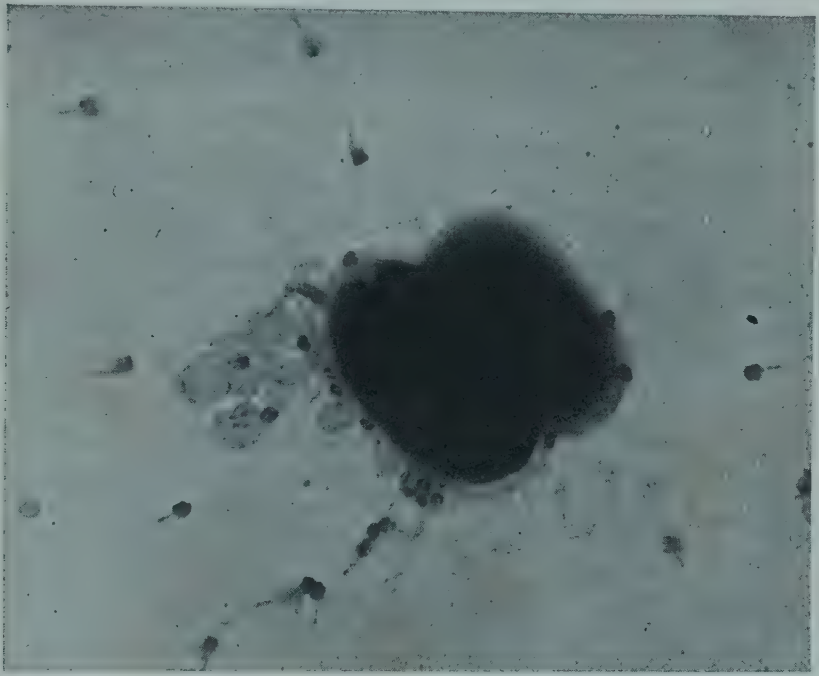

Fig. 389. An early stage (about 5 minutes) of bacteriophagy showing bacteriophage particles apparently converging upon and coming into contact with, 2 cells of *E. coli*. Disc-like particles of already-destroyed cells are clearly visible, as well as fragments of flagella. Note that some of the 'phage particles seem already to have penetrated into the slime layer or cell membrane and to have begun to multiply. (Reduced 1/3 from a photograph representing a magnification of about 30,000 ×.)

A most informative series of electronographs was published in 1948 showing the attack of bacteriophage on bacteria, the multiplication of the 'phage within the cell, and the final dissolution of the cell.[7, 9]

Application of improved electronograph techniques previously mentioned (page 57) in studies of the process of bacteriophage lysis have been carried out by Dr. James Hillier of R. C. A. in collaboration with Dr. Stuart Mudd and Mr. Andrew G. Smith of the University of Pennsylvania. *E. coli* are cultivated on thin collodion membranes so that small, isolated colonies originating from one or two cells are obtained after two to four hours incubation. Each colony is then inoculated with a single bacteriophage particle (on an average) by using a very dilute 'phage suspension. The progress of the attack of the bacteriophage upon the bacteria may be followed by making a series of electronographs. A selection of such pictures is shown in figures 389–392. These pictures are reproduced here through the courtesy of the workers referred to above.

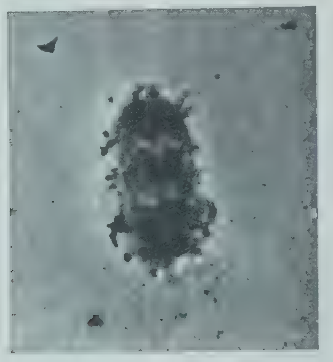

Fig. 390. A somewhat more advanced stage showing multiplication of the 'phage and beginning dissolution of the cell.

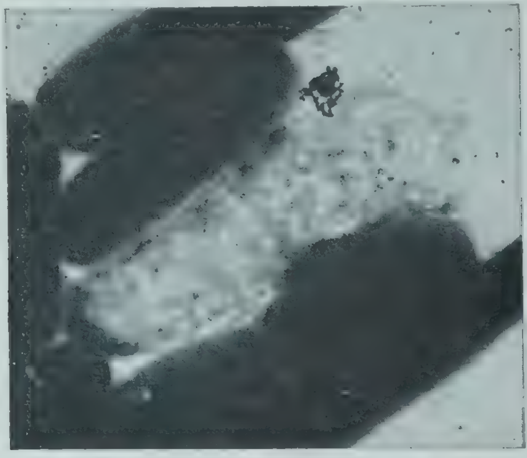

Fig. 391. A group of *E. coli* cells, the central one of which is lysed by bacteriophage. This represents about 30 minutes of action by the bacteriophage. Note that the cell is much swollen. It has probably been broken by the manipulation incidental to making the electronograph. Because, as in this picture, the 'phage particles inside the cell are often small and do not appear to be undergoing division, a question has arisen as to the method of multiplication of the 'phage: whether by a synthetic process involving a sort of molecular rearrangement of the bacterial cell contents; or whether by a process similar to fission as seen in bacteria or analagous to schizogony as seen in malaria parasites (see Chapter 51). (Reduced 1/3 from a photograph representing a magnification of about 15,200 ×.)

Another series illustrates specificity or the mutual interference of two *Escherichia coli* bacteriophages.[8] Viruses, T_1 and T_2, differing morphologically, were used. When both were added simultaneously to a certain culture of *E. coli,* 'phage T_2 could be seen in electronographs to exclude T_1 from the bacterial cells, although both 'phages initially came into contact with the bacteria.[9]

Although only recently introduced into the field of microbiology, phase microscopy has already made notable contributions. An interesting one is the clear demonstration of *living* bacteriophage particles, hitherto demonstrable only by the electron microscope in a *nonliving* state or, with difficulty, by the ordinary microscope. By means of the phase microscope the action of 'phage within the cells, the rupture of the cells, etc., can be followed as it occurs before the eye of the observer.[12]

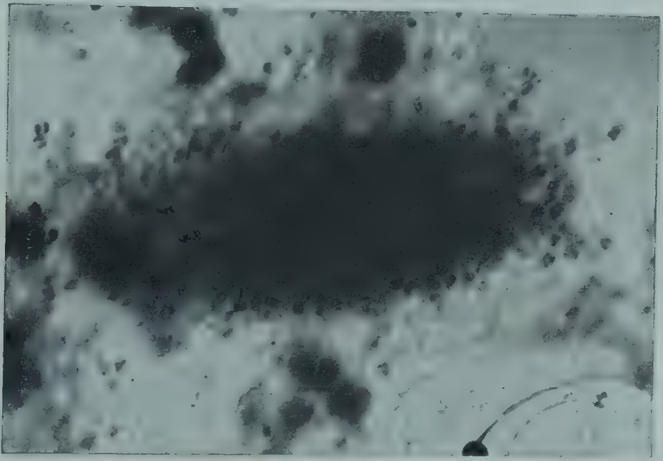

Fig. 392. *E. coli* after approximately 18 hours incubation with bacteriophage, showing a cell completely filled with well-developed 'phage particles and at the point of rupture, with liberation of many 'phage particles. (Reduced 1/3 from a photograph representing a magnification of approximately 30,000 ×.)

Varieties of Bacteriophage. Bacteriophages active against various kinds of Eubacteriales have been found, but none has been found active against sulfur-storing, photosynthetic bacteria, Chlamydobacteriales, Spirochaetales or Myxobacteriales.

'Phages active against Actinomycetales are rare. However, an *actinophage* against *Actinomyces* (*Streptomyces griseus*), the organism from which streptomycin is obtained, has been isolated. By means of electronographs some of the particles of this virus appear to have two tails each.[13]

There is said, by some students of the subject, to be *only one bacteriophage* and that, by suitable *adaptation,* it can be made to attack various kinds of bacteria. Others explain this apparent adaptation as resulting from *selective* growth of one species of 'phage in a mixture, in contact with a pure culture of bacteria suitable to only one. This is a matter still in dispute.

Mutation of Bacteriophage. Susceptibility to specific 'phages seems to be closely related to the antigenic structure of the bacterial cell and possibly to that of the 'phage particles as well.

It has been shown that bacteria may alter in susceptibility to 'phage by mutation and that, by "mutation" the corresponding 'phage can readapt itself to the resistant bacterial mutant. It has been shown also that the first point of attachment of 'phage to the cell is by means of some specific "receptor" on the cell surface. When the bacterium mutates to 'phage resistance, it appears that this is due to some change in the cell surface antigens.[14–17]

"Typing" of Bacteria with Bacteriophage. The process of adaptation (or selective cultivation) of bacteriophage may be carried to a degree where a given strain of 'phage is selectively active, not merely on a single species of bacterium but on only certain types or subdivisions of that single species. These subdivisions or types may be distinguishable solely by serological means, as in the case of *Shigella paradysenteriae* or, in the case of *S. typhi,* by no means except susceptibility to the highly specific 'phage. Certain kinds of hemolytic streptococci have also been extensively investigated in similar relation to bacteriophage susceptibility.

'Phage Types of S. typhi. Craigie[18] and others observed that *S. typhi* could be divided into two main subdivisions on the basis of 'phage susceptibility. The one, corresponding to rough or avirulent variants, they designated as W. This variant is insusceptible. The other, corresponding to smooth, virulent variants, they designated as V. This is highly susceptible. Susceptibility of the V variants seems, further, to be closely related to content of Vi antigen. The smooth strains with less Vi antigen are less susceptible. Passage through several mouse intraperitoneal or intestinal infections rejuvenates both Vi antigen content and 'phage susceptibility. The reasons for the relationship between Vi antigen and specific 'phage susceptibility are obscure.

The relationship of 'phage susceptibility, Vi antigen, smoothness and virulence is undoubtedly of fundamental significance but requires further investigation. The general applicability of the principle to other species of bacteria of industrial, veterinary and botanical interest is obvious, and offers a good field for investigation.

In additional studies it was found that the V strains could be further subdivided into several distinct types on the basis of further adaptation of 'phage. By maintaining a given 'phage on a certain selected strain of V form of *S. typhi*, the 'phage becomes so highly adapted or specific for that strain that it will not act (at least *not when diluted beyond a certain concentration of the 'phage called the "critical dilution"*) on other V forms of the same species. In stronger dilutions the selective specificity is masked by an overwhelming action on all V forms. The first highly specific 'phage of this sort was designated as " 'phage A" and the corresponding susceptible V form strain as " 'phage type A of *S. typhi*." By a like process several 'phage types of *S. typhi* of the V form were discovered, and were designated by letters A, B, C, D, E, etc.

Use of Bacteriophage Types. Having prepared several such highly selective and exclusive type 'phages and determined *the critical dilution* of each, studies were made by Craigie, Lazarus and others of the 'phage types of typhoid bacilli occurring in various patients and in epidemics.[19, 20, 21] The method consists in inoculating an agar plate in several areas with young culture of the epidemic typhoid organism to be tested, allowing a few hours' incubation, and then putting a loopful of the critical dilution of different types of 'phage in the center of each area and incubating until there is clear differentiation between the unaffected areas and the part lysed by the 'phage. The type 'phage producing a large, clear zone in the area of growth indicates the type of typhoid bacilli being dealt with.

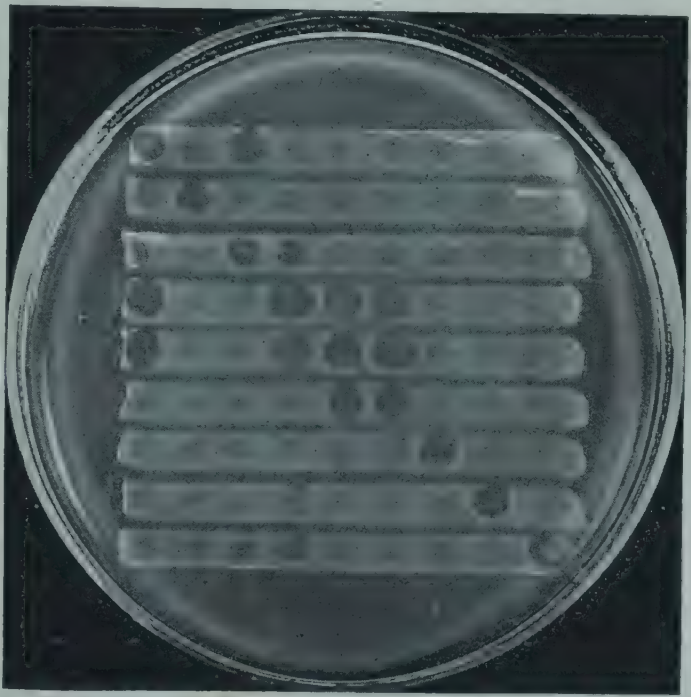

Fig. 393. Large Petri plate containing agar with strips of growth of various species of *Shigella* (inoculated by means of small, sterile camel's hair brushes). Droplets of several different specific bacteriophages diluted to the critical point have been spaced along each strip. The completely cleared area produced by the homologous 'phage, and some partially cleared areas resulting from the action of related 'phages, are clearly seen. (Courtesy of Dr. L. F. Thomen, Ambassador to the U. S. from Republica Dominicana.)

It was possible to show in some instances that the strains of *S. typhi* in a group of patients were all of the same type and therefore probably came from the same source. It was possible to trace the source or sources and to discover carriers of the same type. In other epidemics, two types were found and traced to two different carriers.

Bacteriophage Analysis of Genus Shigella. Related investigations were carried out by Thomen, of the Dominican Republic, a highly skilled scientist.[22] This worker collected strains of important species of *Shigella,* including the serological variants of *S. paradysenteriae* (see section on *Shigella,* page 727), and also several species whose position in the genus is doubtful. By a long process of adaptive and selective cultivations of bacteriophage by methods analogous to those of Craigie and others, but differing in important technical details, he produced a series of 'phages of high differential potency with which an analysis of the relations, serological and cultural, between the various component species and varieties of the genus was made.

The same procedure is applicable to other genera such as *Staphylococcus*[23] and *Salmonella* and possesses certain advantages over serological and cultural methods although it may not replace them at present. The method is rapid, no animals need be immunized, no confusing and expensive carbohydrate tests are needed, and the results seem to be related to serological and cultural properties at the same time.

Nascent Bacteriophage. Evans,[24] at the National Institute of Health, by procedures similar to those described, developed bacteriophages highly specific for certain hemolytic streptococci following observations on this phenomenon by Lancefield.[25] Evans' studies emphasized the importance of what she termed *"nascent 'phage."* In the presence of the specific, sensitive culture on which any given 'phage was developed, the 'phage can attack many strains which are entirely resistant to the filtered 'phage *alone.* Thus, the range of activity of a given specific 'phage for other strains of bacteria may depend on whether the specific homologous culture is present or not.

A protocol for determining the 'phage type of an unknown streptococcus ("x") by the phagological method of Evans[26] is shown below. The 'phage B/563. is specific for culture 563.

Reagent	Tube					
	1	2	3	4	5	6
Culture "x"	1*	1	1	0†	0	0
Culture 563	1	0	0	1	1	0
'Phage B/563	1	1	0	0	1	1

* 1 = 1 drop of culture or 'phage added.
† 0 = nothing added (or 1 drop of saline solution).
All mixtures are made in 5 ml. of broth.

Tube 1 tests the sensitivity of culture "x" to the nascent 'phage B/563 in the presence of the homologous culture 563. Tube 2 tests the sensitivity of culture "x" to the 'phage alone. Tubes 3 and 4 control the viability and growth of cultures "x" and 563. Tube 5 controls the potency of 'phage B/563 for culture 563 and tube 6 controls the sterility of the 'phage. Growth should occur in tubes 2, 3, and 4, but not in 5 or 6. If it occurs in 1, no 'phage action has occurred. If no growth occurs in 1, then culture "x" is of a type susceptible only to nascent 'phage B/563.

The same test can readily be made on an agar plate marked in sectors.

Therapeutic Use of Bacteriophage. When bacteriophage was first discovered, it was thought that disease could be cured with it. If, for example, persons with cholera could swallow cholera 'phage or have it injected into them, it was thought that the 'phage would parasitize the cholera vibrios in the intestine and thus cure the disease. Similarly, it was thought that persons suffering from boils could have antisaphylococcus 'phage injected, or applied in compresses, with a resulting destruction of the staphylococci and cure of the boils. It was soon found that, unfortunately, bacteriophage will not act well in the presence of blood, pus, fecal or any colloidal material.[1, 27] However, Morton and Engley have brought forward evidence that bacteriophage is of prophylactic and therapeutic value in experimental *Shigella paradysenteriae* infections produced in mice by intraperitoneal injections.[28] Natural infections by the oral route appear to be less susceptible to 'phage treatment.

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Chapter 49

THE RICKETTSIAE

Ricketts was first to describe a formerly unrecognized group of microorganisms which differ in many respects from any previously known. His earliest studies on the group were made in connection with Rocky Mountain spotted fever. In 1909 he first described the organisms and mode of transmission of that disease. A year later he discovered the organism of typhus fever while working in Mexico. During his studies of typhus* in 1910 he contracted the disease and died. In 1916, da Rocha-Lima, a Brazilian scientist, made further observations on the organisms described by Ricketts and named them *Rickettsia* in honor of their discoverer. He also gave the name of *prowazekii* to the rickettsiae associated with typhus fever, in honor of another scientist, Prowazek, who had lost his life in the study of that disease.[1] The type species of the group, causative agent of louse-borne typhus fever, is called *Rickettsia prowazekii.*

The rickettsiae constitute a little-understood group of organisms and there is difference of opinion as to whether they are bacteria, stages in the developmental cycle of viruses, or neither of these but an entirely different class of living beings. Most probably they are neither viruses nor bacteria, but an intermediate group. In size they are intermediate between the large viruses, like the lymphogranuloma-psittacosis group, and the bacteria. The 1948 edition of "Bergey's Manual" lists them in the order Rickettsiales, family Rickettsiaceae, with three genera, *Rickettsia, Coxiella* and *Cowdria*. Here we shall use only the general name, Rickettsia, for all of them. There is considerable disagreement as to the proper names, especially of pathogenic species.[2, 3]

Characteristics of Rickettsiae. The organisms are very tiny, having diameters of about 0.3 micron and lengths seldom exceeding 2 microns and usually much less than this. They are variously shaped, like minute rods, diplococci and single cocci. Sometimes relatively long filaments are formed. No spores are produced. Unlike viruses, they are not filterable (with the single exception of *R. burneti*), and in this respect resemble bacteria. They are nonmotile. It is difficult to stain them with ordinary dyes. They can, however, be colored with Giemsa's stain. They are gram-negative. The rod-forms tend to stain more intensely at the tips, somewhat like the Pasteurellas, often giving short rods the appearance of a pair of diplococci (Fig. 394).

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* Do not confuse typhus fever, a blood and tissue disease transmitted by lice and rat fleas, with typhoid fever, a predominantly intestinal infection transmitted by way of the mouth and due to *Salmonella typhi*. The name of typhoid fever was given because the symptoms of that disease were thought to resemble typhus. The word *typhus* means stuporous or drowsy.

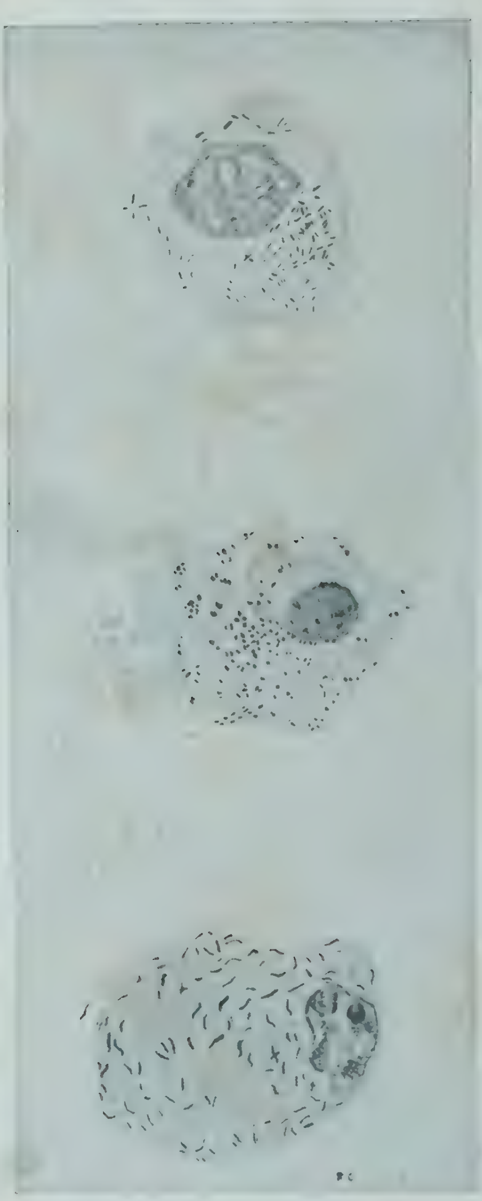

Fig. 394. Rickettsiae in the cells of a guinea-pig (× 1200). (Redrawn from Monteiro.)

Cultivation of Rickettsiae. A few are said to have been cultivated in special lifeless laboratory media but most workers exclude artificially cultivated species from the group of true rickettsiae. Rickettsiae are easily cultivated in live chicken embryos and in tissue cultures like those used to cultivate viruses (see section on methods of cultivation, page 149). They appear to grow best in the cells lining the yolk sac, or in the yolk itself, or both. The yolk grown rickettsiae are widely

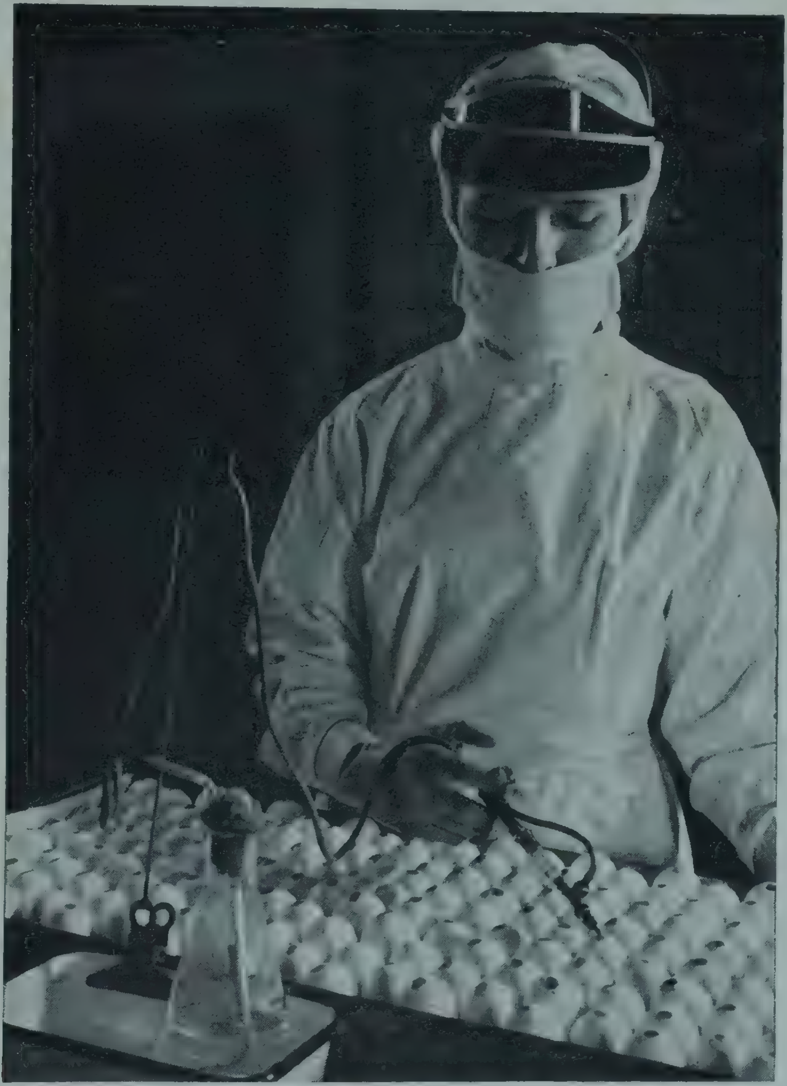

Fig. 395. Carefully dressed technician using a pressure-fed syringe to inoculate fertile eggs with suspension of living rickettsiae. The eggs, after suitable incubation, will be used in the preparation of vaccines or of antigens for diagnostic purposes. (Photo courtesy of E. R. Squibb & Sons.)

used as antigens for complement fixation and agglutination tests, as well as for vaccines.[4, 5, 6]

By extraction of certain lipids from the antigens their specificity is greatly increased, especially with respect to syphilis. However, such egg yolk antigens, containing the whole rickettsial bodies, exhibit some annoying cross reactivity, especially among diseases of the typhus and Rocky Mountain fever groups. By extracting the lipids with ether and then preparing *soluble antigens* by further extraction with benzene and precipitating the soluble products (surface capsular carbohydrates?) of the rickettsiae with sodium sulfate very good non-cross-reacting antigens may be prepared[5a] (Fig. 395).

Habitat. The rickettsiae seem characteristically to inhabit the cells lining the intestines and other tissues of insects, both blood-sucking and non-blood-sucking. Human pathogenic species of rickettsiae inhabit insects which bite man or animals or both. Nonpathogenic rickettsiae have been found in ticks, fleas, lice, bedbugs, spiders and mosquitoes. Some varieties of the organisms are found in the lumen of the intestines of the insects but many are found only in the epithelial cells lining the intestine and in the salivary glands, whence they may be transmitted to man. Since they sometimes occur in the intestinal contents of blood-sucking insects they may appear in the feces and transmission to animal hosts may be obtained by rubbing or scratching this material into the skin.

TABLE XXVI

RICKETTSIAL DISEASES

Disease Types	Causative Agents	Insect Vectors	Weil-Felix Reaction			Chief Locale*
			OX19	OX2	OXK	
Typhus						
endemic	*R. mooseri*	rat flea	+++	+	—	U. S.
epidemic	*R. prowazekii*	body louse	++++	+	—	Europe
Rocky Mountain spotted fever	*R. rickettsii*	sheep, rabbit and dog tick	++	++	—	U. S. Brasil
Tsutsugamushi	*R. orientalis*	akamushi mites	—	—	++++	Japan
Q fever	*R. burneti*	probably ticks, etc.	—	—	—	U. S., Europe
Rickettsialpox	*R. akari*	mites on mice	—	—	—	New York City

* The areas given are those at present of chief interest but are by no means as extensive as is usually the case (see text).

Rickettsial Diseases. The human rickettsial diseases, except Q fever and rickettsialpox, are clinically similar, characterized by fever, skin rashes or dark blotches due to lesions of the blood vessels, and involvements of the brain. However, there are also striking clinical differences between the various kinds of rickettsial diseases. They (and their respective causative rickettsiae) may be divided into five groups on the basis of clinical data, insect vectors, locality, serology and other factors (see Table XXVI).

Both man and certain animals are susceptible to diseases of all five groups, and various biting insect vectors, other than those specified, may transmit each, especially those of the tsutsugamushi, typhus and Rocky Mountain spotted fever groups. Because of the existence of different vectors in different geographical areas, and because there are sometimes clinical differences and differences in geographical distributions, various names have been given to diseases of the various types in different parts of the world. For example, the typho-exanthematico of São Paulo, Brazil, is probably the same disease as Rocky Mountain spotted fever of Montana. Tabardillo of Mexico is typhus, and mite fever of Sumatra is identical with, or closely related to, Japanese tsutsugamushi.

TYPHUS FEVER

This disease has been known since very ancient times. Typhus is found in Central Europe and occurs also in South and Central America, Asia, Russia, Africa and in the United States.[7] It became widely epidemic in occupied countries of Europe during the recent war, a case of history repeating itself.[8]

In the winter campaigns of 1941–1942 and 1943 with little bathing and little chance of removing, cleaning or changing clothing, due to continued fighting, winter cold, and movement of troops and prisoners across Europe in filthy conveyances, lousiness appeared and with it typhus, which spread widely in areas hitherto free from it—Germany, Occupied Europe, etc. The danger of pandemic typhus in Italy and other U. S. occupied areas was averted by the prompt use of DDT generously applied to kill lice. It becomes epidemic whenever conditions are suitable for populations to become thoroughly infested with lice. It may be comforting to know that lice also die of the infection. Both Napoleon and Hitler had cause to know "General Typhus" and "Corporal Louse" to their sorrow. Typhus has killed more soldiers and defeated greater armies than Napoleon ever mustered, and is one of the diseases of great historical importance, like plague, cholera and yellow fever.

Typhus fever is characterized by an onset of about two days during which there is nausea, headache, dizziness and high fever. There then appears a rash which may cover the whole body. It lasts for a week or more and disappears slowly. The patient is lethargic and delirious (typhus is from a Greek word meaning stupor). The mortality is sometimes as high as 70 per cent, but may be as low as 5 per cent. The blood of patients is infectious but the organisms have not been seen in the blood.

Murine (Rat) Typhus. The rickettsiae of typhus fever were found in rats by Dyer et al. after investigations by Maxcy[9, 10] showed that louse transmission of typhus fever from man to man in this country was a negligible factor and that rats were undoubtedly a reservoir of the disease. It was shown that, analogous to plague, the disease was commonly maintained among the rodents through the agency of rat fleas and rat lice. It is transmitted to man from rats by rat fleas (*Xenopsylla cheopis*) when, as in plague, rats and man live in close and continuous contact. Once established in man, the louse-to-man epidemic cycle is said to occur if conditions of lousiness are suitable. By carefully planned and thoroughly executed rat-control campaigns, it has been possible to eliminate this disease from certain areas in this country where it only recently took a large toll of life.[11, 12]

The Weil-Felix Reaction. In 1915 Weil and Felix found that serum of patients with typhus fever agglutinates certain strains of *Proteus* bacilli, especially a strain of Proteus designated as X 19. The reaction is quite specific except: (1) serum of patients with infections by *Proteus* will also agglutinate the organism; and (2) only the aflagellate, or O, variant gives satisfactory diagnostic results. Diagnosis of typhus by means of agglutination tests with OX 19 strains of *Proteus* has for years been a standard procedure but has more recently been supplemented by complement fixation tests using specific rickettsial antigens derived from egg yolk sac cultures of the rickettsiae.[13–16]

The relationship of *Proteus* bacilli to typhus and other diseases caused by rickettsiae appears to depend entirely on the fact that both organisms contain a certain antigen in common. *Proteus* is not a part of the "life cycle" of rickettsiae nor is it a cause of typhus or in any other way related to the disease. Certain other strains of *Proteus* are agglutinated by serum of patients with other rickettsial diseases as noted in Table XXVI.

ROCKY MOUNTAIN SPOTTED FEVER

This disease resembles typhus clinically in important respects, but also differs, so that clinical distinction can be made in typical cases. There appear to be at least three forms of the disease, very closely related: the eastern form, occurring in the Atlantic and other eastern states, less frequently fatal (varying around 25 per cent), and transmitted chiefly by the dog tick *Dermacentor variabilis;* a more highly fatal (varying around 70 per cent) western (Montana and neighboring areas) form transmitted to man by the bite of both male and female sheep ticks (*Dermacentor andersoni*); and the Brazilian form (typho-exanthematico) transmitted by *Amblyomma cajennense*, a tick much like *D. variabilis.* The ticks occur infected in nature, probably maintaining the disease among dogs, rabbits and sheep by their bites, and, in the case of the western form at least, among themselves by transmission of the infectious agent from females to their progeny through the eggs and by sexual contact.[17]

It has been shown that there is a rather close correlation between prevalence of Nuttall's cottontail rabbit and Rocky Mountain spotted fever in the western United States.[18] It is also of interest that dogs apparently become infected, can act as a reservoir of the rickettsiae, and serve as a source of infection for man.[19]

The complement fixation test is far more specific than the Weil-Felix test in all rickettsial infections. The latter tends to appear early and to disappear rapidly after recovery, while complement fixation appears later, as a rule, but can usually be demonstrated much longer afterward.

Any accepted complement-fixation technique may be used, the only distinctive feature of this test being the nature of the antigen, which consists of a suspension of the specific rickettsiae. An excellent technique has been described by Bengtson, who devised the method.[13] Rickettsial suspensions for the antigens are best derived from infected yolk sacs.[6a, 14] Typhus vaccines have been used successfully as antigen in studying typhus fever.[15]

In studying the distribution of endemic typhus in wild rats, Brigham and Bengtson found that in rats in which the infecting agent was demonstrated by actual isolation the complement fixation test was positive in 25 of 26 instances, as compared with 5 positive Weil-Felix tests among 30 rats examined.[16] The complement fixation test shows little or no confusion between Rocky Mountain spotted fever and typhus.[6a] The test is equally valuable in studies of rickettsialpox and Q fever, in which the Weil-Felix test is negative.

Avoidance of Spotted Fever. This is not difficult if one can follow four simple rules:

1. Stay out of tick-infested woods and fields, during May, June, July and August (North and Central states), especially where there is dense undergrowth overrun with wild mice, rabbits, etc.
2. If forced to travel in such areas wear high boots and white or light-colored clothes made of smooth, nonwoolly goods. Wear socks or boots outside of trouser legs because ticks tend to climb upward. They can often be felt on the neck after a while.
3. Examine the clothing (inside and out) and body twice a day. If ticks are found, do not become unduly alarmed. It is unlikely that your tick is infectious; they do not bite immediately; even after biting they do not always infect immediately because the virus requires from two to eight hours' contact with your blood in the tick to become "activated" unless the tick has been

warm for weeks or partly engorged on other animals, when it is already active. If the tick has taken hold, remove it and touch the area with iodine. Removal may be accomplished by gentle traction, unless the head is well embedded in the skin. Force may detach the head and leave it in the skin. With a fine forceps pinch up the epidermis, containing the tick's head, into a tiny fold and clip off just enough to remove the head. Disinfect the spot thoroughly. Do not crush or damage the tick or use bare hands. Another simple method consists in striking an ordinary household match, shake it out as soon as it flares, and apply the hot head to the rear end of the tick; he will quickly withdraw his beak. Do not apply the flaming match, for this will kill the tick before he can withdraw.

4. Do not remove ticks from dogs or other animals or handle ticks from dogs or come into contact with crushed ticks. Keep dogs outdoors in the tick season.

TSUTSUGAMUSHI DISEASE

This disease, resembling typhus clinically, is found principally in Japan, Malaya, and islands of the South Pacific.[20] The disease occurs most frequently in areas covered with scrubby undergrowth, and in swamp and flood areas. The common transmitting agent (*Trombicula akamushi*) is much like the American "chigger." It is a mite of red-orange color, almost microscopic in size, which infests field mice and other animals in rural areas. The rodents serve as a reservoir for the disease. The larvae are infected by biting infected mice and transovarially. They transmit the rickettsiae to man and animals by their bite. The adults do not bite mammals, but may live on plant juices. The disease is largely restricted ordinarily to low-class laborers and squatters who inhabit the areas described and is more common in males who labor in the fields; for example, rice planters. It was a problem of considerable importance during U. S. military operations in the Pacific theater. The monumental studies of Blake, Maxcy, Sadusk, Kohls, Bell, Armbrust, Irons, Philip and others under fire in 1943 with the United States Army should be consulted for complete details on all phases of the subject.[21]

The disease begins with a hard swelling or ulcer of the skin at the site of the bite. Then come fever, chills, a local ulceration, nausea, headache and the rash. The mortality may run as high as 60 per cent, especially in older people. The serum of patients agglutinates only the OXK strain of *Proteus*. The hard swelling at the site of the bite is also found in fièvre boutonneuse and tick-typhus of South Africa, two diseases closely related to typhus fever, but tick-borne. The Weil-Felix reaction in these two diseases is like that in the Rocky Mountain fever group. The complement-fixation test is highly specific in tsutsugamushi. Ether-extracted yolk-sac preparations are especially good antigens.[6a]

Q FEVER

In 1935 Derrick, in Queensland, Australia, studied a febrile disease resembling influenza or "grippe" among workers in stockyards, dairies, etc. Blood and urine of the patients injected into guinea pigs produced an illness followed by immunity in the recovered animals. The disease was designated "Q" as being of Questionable identity; hence the unfortunate term "Q," which has persisted.* Burnet later found monkeys and mice susceptible and rickettsiae of a distinctive type were found in mice.

In 1938 Davis and Cox reported on a *filterable* infectious agent in ticks found at 9-mile Creek, Montana. A famous scientist, visiting the laboratory for a few hours, contracted the disease ("9-mile fever") and from him was isolated a rickettsia identical with the Q fever strain, which is now called *R. burneti*. His serum showed no cross serological relationships with other rickettsial diseases except Q fever. During studies of the organism in the National Institute of Health, an epidemic of the disease occurred among 47 workers in that institution as well as in many guinea pigs.[22] The mode of transmission is not known with certainty.

In 1944 several outbreaks of influenza-like disease occurred among United States troops in Italy and other outbreaks have been reported in the Balkans ("Balkan grippe"); Panama; Chicago; Amarillo, Texas, etc. The rickettsiae from all of them, isolated by guinea pigs or mouse inoculation, have been found identical with *R. burneti*. Diagnosis has been confirmed by agglutination and complement fixation tests and cross immunization tests.[23, 24] The disease may be quite common, masquerading as "flu," or atypical pneumonia, all over the world.

The mode of transmission is not known. It is clear that association with cattle, as in abattoirs or in stockyards and dairies, constitutes a distinct hazard in respect to Q fever. But whether infection is transmitted by insects, dust, dung or handling flesh, or all of these, is not clear. Cattle do not show any evidence of being infected or ill.[25] *Rickettsia burneti* has been found in milk. How they get into the milk is not clear, but the probability is that infected cows are responsible. Drinking the milk does not seem to transmit the disease to human beings, but transmission to man from raw milk does, nevertheless, seem to occur. Pasteurization kills the organisms.[26]

The transmission of Q fever presents even a prettier mystery than the transmission of poliomyelitis.

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* According to some sources, Q stands for Queensland.

RICKETTSIALPOX

This febrile disease was first described during 1946 in New York City, particularly in a certain group of apartment houses there. It is transmitted by a mite (*Allodermanyssus sanguineus*) which bites mice and human beings. The mite is harbored by house mice and frequents walls of warm rooms and spaces such as the outside of the warm (not hot) incinerators in the buildings mentioned. Mice frequent the incinerator rooms to eat scraps of garbage, and also go into the living apartments and even beds of the inhabitants. The rickettsiae, called *R. akari,* have been isolated from the blood of patients, mice and mites.

Serum of patients and of mice gives positive complement fixation tests with the rickettsiae. There is no confusion with other rickettsial diseases, except for reactions with Rocky Mountain spotted fever antigens if the sera are not sufficiently diluted.

Clinically, the disease begins much as tsutsugamushi, with a small ulcer at the site of the mite bite. Later there develops a disease somewhat like Q fever. However, a rash appears, suggestive of tsutsugamushi, etc., but not so extensive. The spots later vesiculate and the disease has been mistakenly diagnosed as an "atypical chicken pox." The incubation period is about ten days and the fever, which may reach 104° F., may last ten days.[27, 28] Mortality is low or nil.

That rickettsial diseases are more common than is generally suspected is suggested by the description of two other new diseases, Colorado tick fever and North Queensland tick typhus.[29, 30, 31]

RICKETTSIA VACCINES

One of the oustanding results of studies of Rocky Mountain spotted fever was the development of an effective and safe preventive vaccine by Spencer and Parker of the United States Public Health Service.[32] This is made by grinding up heavily infected ticks and treating extracts from them with 0.5 per cent phenol to disinfect them and to inactivate the rickettsiae.

Because of the spread of typhus in Europe and Asia in 1942 to 1944 extensive studies have been made of vaccination against the disease. Some of the vaccines tested have consisted of living rickettsiae of murine typhus, but these are falling into disuse because of the danger of spreading the disease[33] and because the killed vaccines seem equally effective.

A typhus vaccine recently under trial in foreign field service under United States jurisdiction was originated by Cox.[34] The rickettsiae are injected into the yolk sac of developing chick embryos, where they multiply enormously (Fig. 395). The vaccine is prepared from either the whole embryo, or from the yolk sac alone, by grinding and adding 1.0 per cent phenol and 0.5 per cent formalin. Much of the solid tissue is removed by slow centrifugation of the ground-up embryo. The rickettsiae are then collected and washed by several high speed centrifugations. This procedure is representative of the preparation of various sorts of rickettsial vaccines. Recent improvements have included extraction of fat from the vaccine-egg mixture with ether. These vaccines seem to give promising results,[35] but certain, durable, and absolute protection of each individual must not be expected. Vaccines against other rickettsial diseases have been prepared on a large scale in the same way.

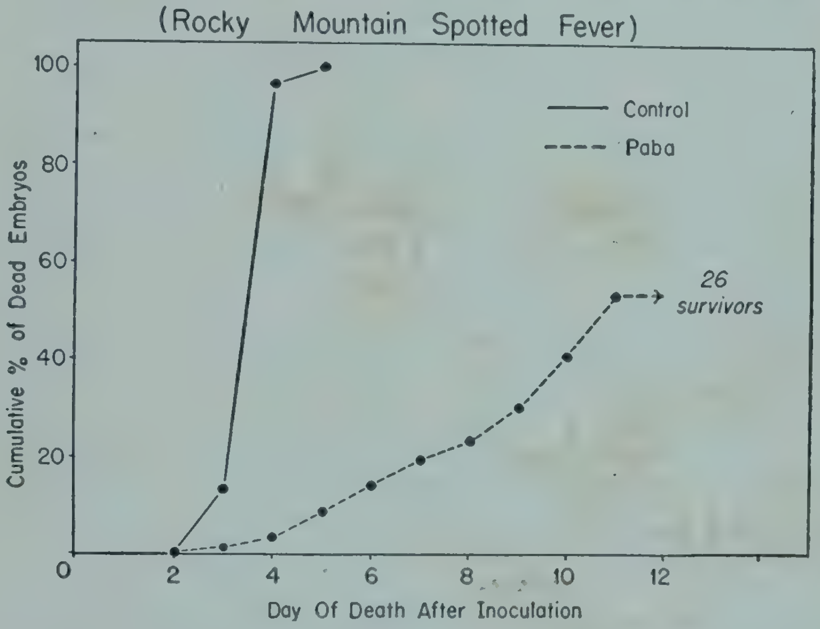

Fig. 396. Effect of Paba on time of death. (Hamilton, Howard L., in Proc. Soc. Exper. Biology and Medicine, vol. 59.)

PABA. These letters stand for para-aminobenzoic acid which has been found to give brilliant results in the treatment of several rickettsial diseases. Clinical discussions are beyond the scope of this book, but this particular therapeutic agent is mentioned because of interest attached to its mode of action. On page 801 it was pointed out that disturbances of metabolism of the host cell militate against growth of viruses. PABA is known to play an intimate role in cell metabolism, especially in relation to respiration and the action of coenzymes (see page 294). Its therapeutic action in rickettsial infections seems not to be directly on the parasites, since they are all intracellular, but more likely upon the host cells. However, further study is to be made of this question. It is extremely fundamental in chemotherapy.[36–41]

Figure 396 shows a comparison of deaths among chick embryos infected with Rocky Mountain spotted fever rickettsiae, some treated with PABA and others not treated.

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Chapter 50

THE PLEUROPNEUMONIA-LIKE ORGANISMS

The organisms of the pleuropneumonia group are definitely not protozoa or algae, yet are difficult to classify as bacteria, viruses or rickettsiae, since they possess some of the properties of all, yet not all the properties of any. The first member of the group was demonstrated in 1898 by Nocard and Roux, who cultivated it on cell-free media from serofibrinous exudate in the pleural cavity of a cow suffering from the disease now known as pleuropneumonia of cattle. In cultures, the minute, round granules, not unlike the elementary bodies of the psittacosis organisms appeared to send out long filaments radiating in various directions and often branching, like *Streptomyces*. Borrel named the organism *Asterococcus mycoides*. When filtrates from pleuropneumonia cultures and tissues were tested, they were found to contain the organisms in viable form.

Cultivation. Growth of filterable organisms related to *Asterococcus mycoides* will occur on suitable medium, even though no bodies resembling rickettsiae or ordinary bacteria are visible in Giemsa-stained smears of the inoculum. A very favorable medium is heart-infusion peptone agar, *p*H about 7.7, containing about 25 per cent serum. Horse, bovine, human or rabbit sera will serve but an organism once adapted to growth on the protein of one animal may sometimes be induced only with difficulty to utilize that of another animal. Media containing less than about 5 per cent of serum will rarely support growth of pleuropneumonia organisms, evidence of a striking difference between these forms and true bacteria. Plates or tubes of fluid medium may successfully be inoculated with minced infected tissue, exudate, swabbings, etc. Growth occurs more readily, as a rule, on solid media. Incubation is at 37° C. for from two to ten days or more and the atmosphere in the plates must be saturated with moisture. The organisms are facultative with regard to oxygen. Sulfonamide drugs and penicillin are often used to suppress growth of extraneous bacteria when isolation is attempted from material heavily contaminated with ordinary bacteria. Certain special precautions in cultivation are described by Dienes.[1]

Colonies. Minute colonies, so small as usually to be invisible to the naked eye and visible only with a hand lens or low power of the microscope, appear along the lines of inoculation on plates (Fig. 397).

These must not be confused with similar-looking structures ("pseudocolonies")[2] composed of sphero-crystals and other aggregations of lifeless material which sometimes appear on the surface even of sterile serum agar plates as a result of various physico-chemical factors acting locally, including scratching with a needle, and which have no relation to the inoculum except that scratches on the surface seem to facilitate their formation (Fig. 398). The genuine colonies are definite in outline and slightly raised and have a dark, vacuolated or granular central portion.

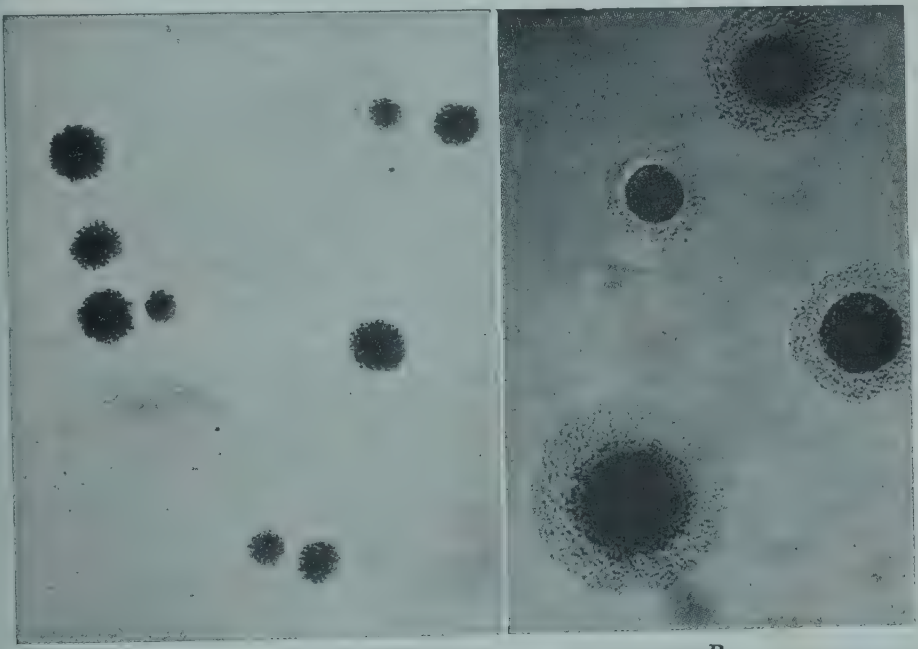

Fig. 397. Colonies of pleuropneumonia-like organisms (L bodies) on 20 per cent horse-serum dextrose-starch agar. (Forty-eight hours at 37° C.) (× 70.) Note the minute size and granular structure. *A* and *B* are from different cases of rat-bite fever. (Brown and Nunemaker, Bulletin of the Johns Hopkins Hosp., vol. 70.)

The agar containing such a colony may be cut out and used to inoculate fluid medium (25 per cent serum-infusion-broth, *p*H 7.8 to 8.0) or other plates by sliding the piece of agar, inverted, over the surface of fresh medium.

The central density of colonies on solid medium is due to growth of the organisms below the surface of the agar, somewhat like Actinomycetales. The central portion above the surface contains well defined elementary bodies which may be stained in situ.

Staining. The best method of staining is to cut out a piece of agar on which the colonies are growing and invert upon it a coverslip on which is a deposit of methylene blue and azure from an alcoholic solution dried there. The microscope is focused on the coverslip. The elementary bodies in the central part of the colonies are seen to be minute, round or elongated forms, ranging downward from about 0.2 μ. They may also be ring-shaped; sometimes bacillary, but exceedingly minute. Later these swell to round or oval bodies 10 μ or more in diameter, which are part of the reproductive cycle. The

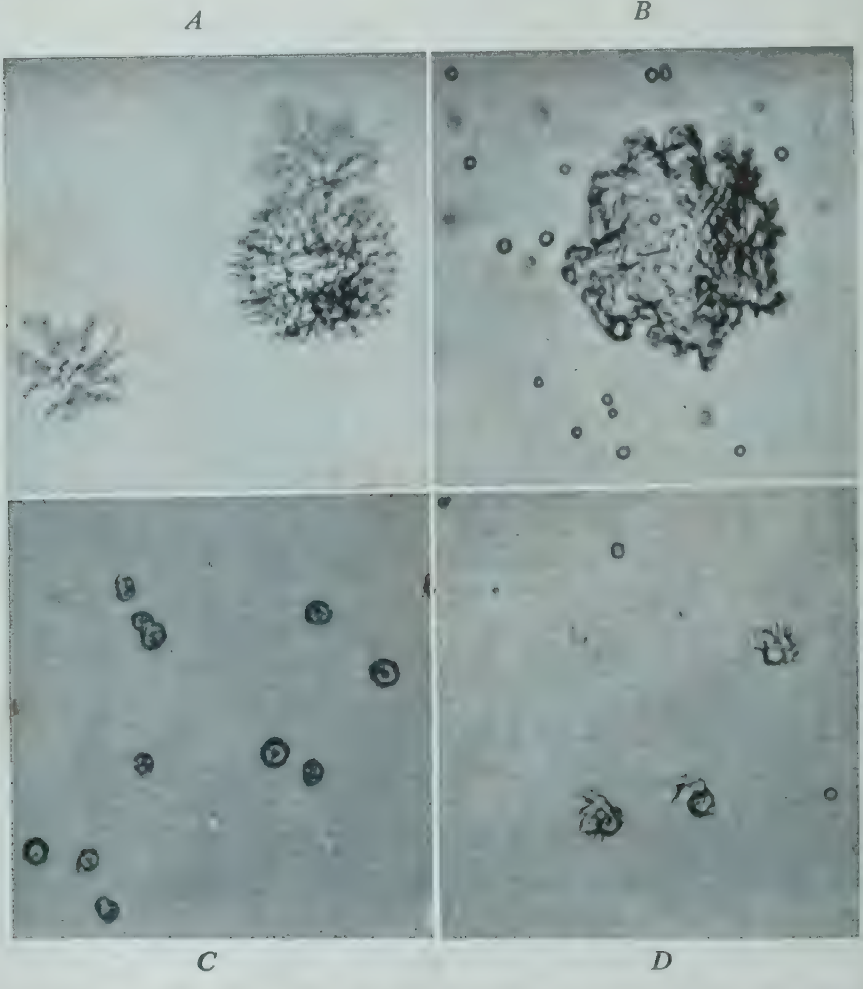

Fig. 398. *A*. Pseudo-colonies in 30 per cent rabbit serum agar, incubated two weeks. × 115. *B*. Thirty per cent rabbit serum agar, incubated six weeks. Pseudo-colonies have marked chiseled appearance. × 115. *C*. Thirty per cent ascitic fluid agar, incubated two weeks. Pseudo-colonies in form of small discs with central depression. × 115. *D*. Thirty per cent beef serum agar, incubated one week. Small central concave disc with curved peripheral areas forming cocks-comb pseudo-colonies. × 115. (Brown, Swift and Watson, J. Bact., vol. 40.)

periphery of the colonies is often made up of large round bodies which may stain distinctly; or these bodies may have degenerated and coalesced into a foam-like web which does not stain well and appears to represent sacs, empty or filled with granular fluid. These are not viable.[3]

Life Cycle. The fundamental units of growth in the colonies are the tiny elementary bodies. These may grow into pleomorphic filaments which again fragment into elementary bodies. The elementary bodies may also swell to form large, round bodies. Inside these large bodies more elementary bodies form, which burst forth and continue the cycle. In the organism of bovine pleuropneumonia filaments may be sent out from the large round bodies as well as from elementary bodies. The elementary bodies of any species may also multiply as such. This or similar types of life cycle, including the appearance of L bodies (described below) have been found in certain bacteria, like *Bacteroides,*[4] *H. influenzae,* gonococci and *Past. tularensis.*[5, 6, 15, 16, 17] Some very curious bacillary swellings have also been observed in cultures of *Escherichia coli* treated with penicillin. These are suggestive of some of the cyclical stages described above.[7] They may be merely involution forms of *E. coli.* A curious phenomenon suggesting part of the life cycle of the pleuropneumonia-like organisms has also been described in cultures of *Proteus* organisms. In some cultures of this species filaments of bacilli are produced. When these meet the filaments of certain other strains of *Proteus,* a mutual reaction occurs in which the filaments swell up and may reproduce as either ordinary bacilli or pleuropneumonia organisms.[8]

A number of very fine micro electronographs showing these various forms, both in a typical pleuropneumonia organism, and as occurring in a culture of ordinary bacteria, have been published by Smith, Mudd and Hillier.[8a] The significance of these various forms is more fully discussed on page 876 in the section on classification. A structural detail of especial significance is the fragility of the cell membrane of pleuropneumonia organisms, and of bacteria (*Bacteroides, Past. tularensis,* etc.) in which pleuropneumonia-like phases occur.

Varieties of Pleuropneumonia-like Organisms. Following its discovery by Nocard and Roux in 1898 the cause of bovine pleuropneumonia was for some years classed with the viruses because of its minute size and filterability. Several similar organisms were later discovered and all are now classed as the Borrelomycetaceae or pleuropneumonia group.

Agalactia is a disease principally of sheep and goats and causes an inflammatory disease of the mammary glands of lactating females which stop secreting milk—hence the name of the disease. The lesions of agalactia are not confined to the mammary glands however, the infectious agent attacking especially the joints and occurring in secretions of the eyes and in the blood. In 1923 Bridre and Donatien cultivated from the joint fluid of an infected sheep an organism very closely resembling that discovered by Nocard and Roux. It differs from the latter only in its pathogenic and serological properties.

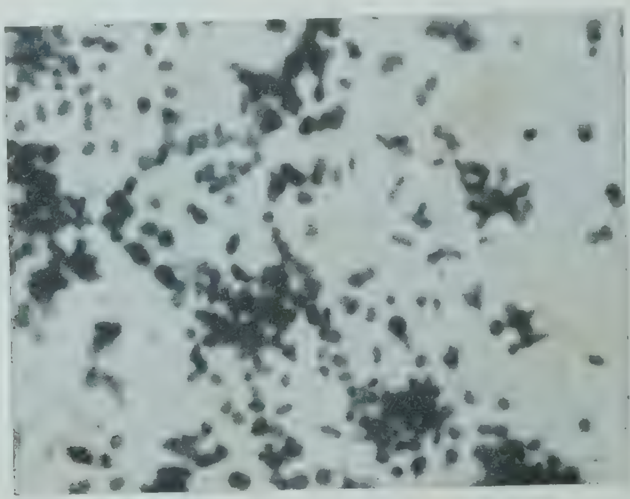

Fig. 399. Slightly disturbed young colonies in which rodlike and ring forms are apparent. Giemsa staining after agar fixation. × 3,000. (Dienes, L., in J. Bact., vol. 50.)

Asterococcus canis. A similar infectious agent was isolated by Shoetensack in 1934 from nasal discharges, lung exudate, etc., of dogs suffering from distemper. The relationship of the agent to distemper is obscure, as distemper is caused by a well-known virus. Possibly there is a symbiotic activity. Shoetensack called his organism

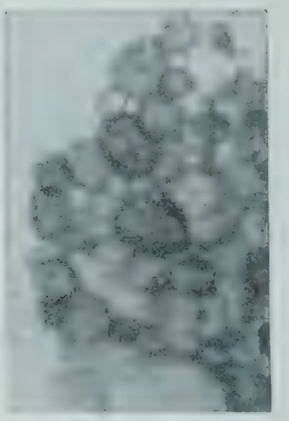

Fig. 400. Fully developed colonies of pleuropneumonia-like organisms on ascitic agar plates inoculated from the uterine cervix. Wet stained agar preparation showing the large bodies and the empty blebs produced by vacuolization of the large bodies. × 900. (Dienes, L., in J. Bact., vol. 50.)

Asterococcus canis because of its apparent similarity to *Asterococcus mycoides,* the organism of bovine pleuropneumonia.

Streptobacillus moniliformis. One of the most interesting members of this curious group of organisms is generally referred to as "L_1" following the nomenclature of Klieneberger[9] who first described it in 1935. It is present in nearly all cultures of the gram-negative, very pleomorphic, somewhat actinomyces-like bacterium usually called *Streptobacillus moniliformis.* The streptobacillus is believed by some to be merely a variant form of the L_1 organism with which it is associated.[10] The name *Asterococcus muris* has been proposed for L_1.

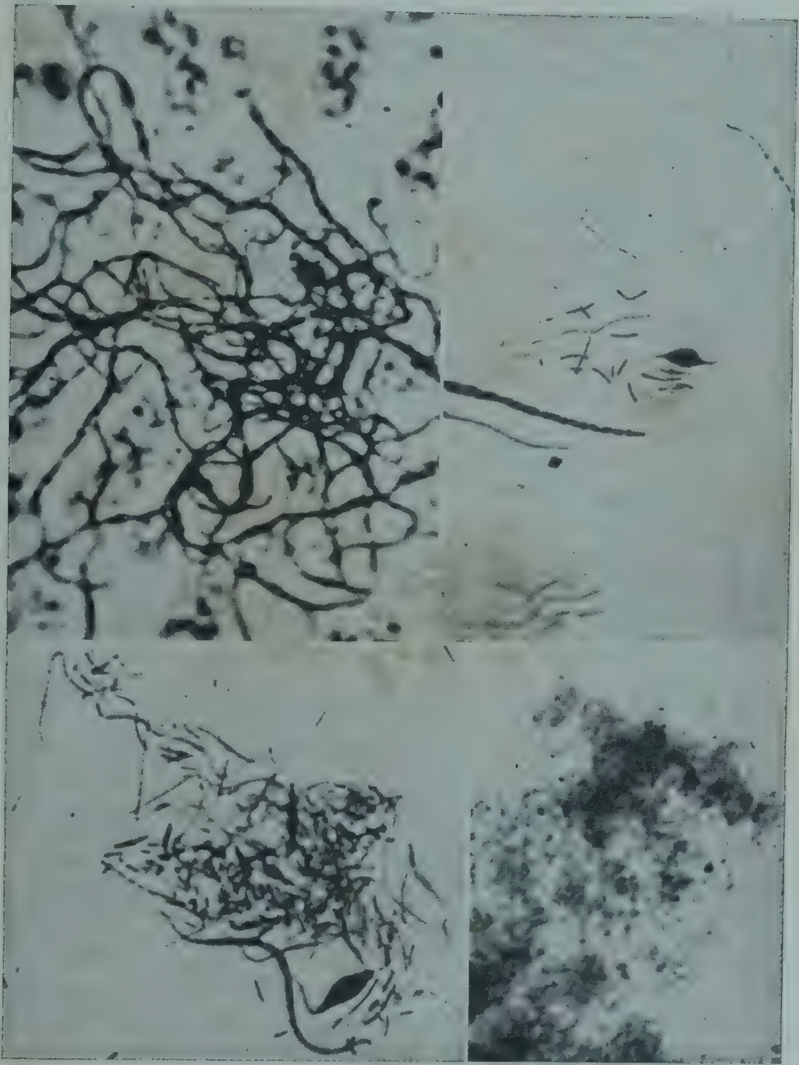

Fig. 401. Various forms of *Streptobacillus moniliformis.* (Brown and Nunemaker, Bulletin of Johns Hopkins Hosp., vol. 70.)

S. moniliformis and the L_1 organism grow well under the same conditions as other *Asterococcus* organisms. *S. moniliformis* may be obtained from pus of the ears of rats where it frequently causes otitis media, and from the blood of human beings suffering from rat-bite fever, one type of which it also causes.[11, 12, 13] The colonies, superficially examined, are much like those of ordinary bacteria. Smears show gram-negative rods and filaments in which branching is common. Swellings, globules and granulations of the cells are a prominent feature as the colonies age. The globules contain smaller elements which may be liberated by crushing under a coverslip (Fig. 401).

"L_1 Bodies." After several days of incubation of the colonies, dense areas appear under them and, by washing away the earlier growth of *S. moniliformis* with broth, the denser portions are found to remain adherent to the agar. These are the growths of the L_1 elements. The agar containing these elements may be cut out, macerated with broth and used to inoculate new agar or broth. Their life cycle is like that of *Asterococcus mycoides*. On agar they tend to retain their L_1 form; in broth the filamentous growth of *S. moniliformis* often reappears but not always. A great question remains unsettled as to whether the two forms are variants of each other, or whether they are distinct species.[14] One view is that L_1 organisms are analogous to the pleuropneumonia organisms, but that in the latter the ability to form the filamentous growth like *S. moniliformis* is permanently lost (or was never developed). In the L_1 forms, according to this view, it is still present or represents a slightly more advanced evolutionary phase which is more marked and persistent than in others. Any student of the group will find here a fascinating problem, challenging the objectivity of his judgment, his technical skill and his experimental ingenuity.

The elementary granules, or some other reproductive form, will pass very fine filters, and *S. moniliformis* colonies will sometimes develop from such filtrates. True bacteria do not pass such filters. However, before one could be certain that *S. moniliformis* developed from the filter-passing L_1 reproductive units, one would have to be very sure of the integrity and fineness of his filters. These studies should be facilitated by the use of penicillin, which prevents growth of the *S. moniliformis* but permits growth of the L_1 bodies.

Other Varieties. Certain lung diseases, pyogenic conditions and arthritis of rats and guinea pigs appear to be due to members of the group, some of which have been called "L_2," "L_3," "L_4," "L_5," "L_6," etc. In Europe nonpathogenic, apparently saprophytic, forms have been isolated from London sewage and from soil, leaf mold and dung.[15]

In studies of the virus of atypical primary pneumonia in eggs, a hitherto unidentified agent of the pleuropneumonia group was discovered. Cultures of the organism cause hemagglutination, and produce a toxic substance.[19] Forms similar to L_1 bodies have been found to occur in cultures of several species of bacteria and are generally spoken of as L forms or L variants.

Pathogenic Properties. *Asterococcus mycoides* and a similar organism obtained from mice, produce an exotoxin somewhat like diphtheria toxin (compare with lymphogranuloma organisms).

A curious feature of many of the pathogenic forms is their affinity for joints and eyes. Some have repeatedly been isolated from animals suffering from arthritis.[20, 21] No relation between human arthritis or rheumatism and this group of organisms has been satisfactorily demonstrated. They may also infect numerous unrelated tissues. Each species seems to be definitely restricted to one animal species although the restriction is not absolute. Organisms of the group appear to be fairly widely distributed.

Antigenic Relations. Pleuropneumonia-like organisms from different sources are antigenically active and their immunological relationships have been extensively studied. Injection of cultures into rabbits induces the formation of agglutinins and other antibodies by means of which serological groupings, etc., have been investigated. The strains from bovine pleuropneumonia are serologically uniform and unrelated antigenically to any other group of similar organisms such as those from agalactia of sheep, or from dogs affected by distemper, or from mice, rats, sewage and many other sources. The several groups of organisms are serologically separable and are also subdivided into subgroups.[22]

TABLE XXVII

FERMENTATIVE PROPERTIES OF SOME PLEUROPNEUMONIA-LIKE ORGANISMS

Source	Lactose	Dextrose	Sucrose	Maltose
Pl. bovis	+	+	−	+
Agalactia	±	+	+	+
Others (rats, mice, etc.) . .	Variable	+	−	−

Fermentations. In addition to serological group differentiations certain divisions are possible on the basis of fermentative properties. Thus, the organism of pleuropneumonia bovis is said to differ from that of agalactia in that the former fails to ferment sucrose while the latter causes fermentation. Both ferment maltose, differing from all other strains in this respect, while all known strains ferment dextrose but fail to ferment sucrose except as noted.[23] (See Table XXVII.) The fermentative studies are attended by numerous technical difficulties.

Resistance. In respect to resistance to various unfavorable agents, this group of organisms resembles the non-sporeforming bacteria in many respects, and true viruses in others. Some are very sensitive to heat, 45° C. for fifteen minutes proving lethal to some strains isolated from mice; while other varieties, as that from agalactia, may survive seven but not ten minutes at 53° C. One strain, isolated from rats, may resist 56° C. for nearly an hour. Resistance of most forms to phenol, hydrogen peroxide and ultraviolet light is of a degree comparable with that of ordinary bacteria. They may be preserved for weeks or months by freezing and drying and will remain alive for a month or more on solid media sealed to prevent drying.

Classification. According to Dienes, the pleuropneumonia-like organisms are merely a stage in the life-cycle of bacteria.[3] Smith, Mudd and Hillier[8a] have further clarified this hypothesis concerning the relation of the pleuropneumonia organisms to the true bacteria. According to this view the L or filterable form is merely a variant of the true bacterial form. There is, on the one hand, a group of pleuropneumonia (L) organisms which maintain this minute, L form persistently. These include *Asterococcus bovis,* agalactia strains, L_2, L_3, L_4, etc. On the other hand there are L organisms which occur in cultures of various bacteria (*Streptobacillus moniliformis,* some strains of *E. coli, Bacteroides, N. gonorrhoeae,* etc.). The development of these L variants is part of the life cycle of the true, normal sized bacteria, which parallels the life cycle of the permanently minute pleuropneumonia-like forms. Some of the forms incidental to this life cycle in a true bacterium are seen in the article by Smith, Mudd and Hillier,[8a] while analogous forms in a permanently minute, pleuropneumonia-like organism are also seen.

Especially convincing is the fact that some of these L variants from true bacteria have been shown to revert to the true bacterial form.[27] It is significant, as noted previously, that the bacteria which readily produce L variants have thin, fragile cell membranes.[8a] Some L variants from bacteria revert to the bacterial form only with difficulty or not at all. Many bacteria presumably possess the potentiality of producing L variants but do not ordinarily do so unless some special stimulus, such as penicillin, be present.[27, 28]

It is of especial interest to note electronographs that show pleuropneumonia organisms merely as very minute counterparts of true bacteria, and that in both the true bacterial form and the minute L variant of bacteria (such as *Bacteroides*) the same life cycle is seen.[8a] If this concept be finally established, the pleuropneumonia group cannot be longer regarded as a race apart, but as a form of true bacteria, however their properties may differ from our traditional definitions of bacteria. We shall have to alter our definitions to fit the facts.

Until this relationship is established we may regard them as a separate group, to which Turner has given the tentative name of Borrelomycetaceae.[24] Breed et al. include them in the "Manual of Determinative Bacteriology" under this name.[25] Obviously they are not regarded as bacteria according to present definitions of Schizomycetes. Sabin[26] has proposed the name Paramycetes for the group.

They resemble viruses in size and filterability, and to some extent in pathogenicity but differ in ability to grow on lifeless media. The existence of saprophytic forms definitely excludes them from classification with true viruses.

Certain phases in the development of pleuropneumonia organisms at times bear some morphological resemblance to some forms of rickettsiae, but there the resemblance between the two ends. The rickettsiae are neither highly pleomorphic like the pleuropneumonia organisms, nor are they readily filterable or cultivable on lifeless media or in the absence of living cells.

Pleuropneumonia organisms are not related to the filterable forms of ordinary bacteria (Eubacteriales) which have previously been discussed. The very existence of the filterable bacteria is in considerable doubt. Even if they do exist they are not visible and are not cultivable in the way the pleuropneumonia organisms are. The latter do not revert to ordinary bacillary forms unless *Streptobacillus moniliformis* and one or two other species[8a] be regarded as exceptions.

It seems proper, for the time being, to regard the group as another class of organisms, intermediate between the others.

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Chapter 51

THE PATHOGENIC PROTOZOA

Protozoa are unicellular animals. They hold a position in the animal kingdom analogous to that held in the vegetable kingdom by the bacteria. Although microscopic in size, they are, in general, much larger than the bacteria and their structure and physiological activities are much more complicated than are those of the single-celled plants. Hundreds of kinds of free-living protozoa are to be found in much the same situations in the outside world as the numerous kinds of harmless bacteria. They are common in stagnant pools of fresh water, soil, feces, etc. Infusions of hay incubated a few days usually teem not only with bacteria but with protozoa. Leeuwenhoek was first to observe many of these. Only a few (possibly a score) kinds of protozoa are harmful to man. We shall describe the more important of these very briefly in this chapter. Students interested in learning more about protozoa should consult a book on protozoology.[1, 2] Many are of great importance in water and sewage purification, soil fertility and plant and animal disease.

Structure and Biology of Protozoa. In protozoa there is always a well-defined nucleus which can be clearly seen in stained preparations with the microscope. There is also a definite cytoplasm and cell wall, and there are usually many small granules, and vacuoles filled with clear fluid or ingested food particles. These have various functions connected with motion, digestion and reproduction. The specialized portions of the cell are called "cell organs" or organelles.

Classification of Protozoa. All of the protozoan parasites which we shall discuss are motile during at least one stage of their existence, the means of motility being different in each group. These differences are used as a basis of classification. There are four great groups of protozoa and one or more species in each group is pathogenic. The groups may be listed, with one or two common examples, as follows:

Class I. *Sarcodina* (move with pseudopods).
  A. *Endamoeba histolytica*—causes amoebiasis.
  B. *Endamoeba coli*—not pathogenic.
Class II. *Mastigophora* (move with long, whiplike lashes called flagella).
  A. Intestinal tract:
    (*a*) *Giardia lamblia*—possibly causes enteritis.
    (*b*) *Trichomonas hominis*—not pathogenic.

- B. Urogenital tract:
  - (*a*) *Trichomonas foetus*—a cause of abortion in cattle.
  - (*b*) *Trichomonas vaginalis*—may cause vaginitis.
- C. Blood stream:
  - (*a*) *Trypanosoma rhodesiense*—causes African sleeping sickness.
  - (*b*) *Trypanosoma gambiense*—causes African sleeping sickness.
  - (*c*) *Trypanosoma equiperdum*—causes dourine ("equine syphilis") of horses.
  - (*d*) *Trypanosoma cruzi*—causes Chagas' disease.
- D. Blood stream and tissues:
  - (*a*) *Leishmania donovani*—causes kala-azar.
  - (*b*) *Leishmania tropica*—causes oriental sore.
  - (*c*) *Leishmania braziliensis*—causes espundia or South American leishmaniasis.

Class III. *Sporozoa* (move with pseudopods only in immature stages; male gamete is flagellate).
- A. *Plasmodium vivax* (also *P. malariae, P. falciparum* and *P. ovale*)—cause malaria.

Class IV. *Ciliata* (move with cilia).
- A. *Balantidium coli*—causes enteritis and ulcerations.

Some authors include a fifth class, Suctoria, in which young stages are ciliated while adult stages are provided with tentacles. None of these is known to be pathogenic for human beings.

Reproduction and Life Cycles of Protozoa. Some protozoa multiply both sexually and asexually, others only by binary fission. Unlike the bacteria, the flagellates divide lengthwise (longitudinal fission) rather than crosswise. Ciliates divide transversely. The nucleus undergoes changes very much like mitosis. Some species exhibit a simplified or primitive form of this mitotic phenomenon. One type of mitotic phenomenon, in which the nuclear membrane remains intact, is called promitosis.

Sexual multiplication by protozoa often involves rather complicated procedures. Many protozoa, during their development, pass through a definite and readily demonstrable series of stages and are thus quite different from the bacteria. These series of developmental stages are called *life cycles* and often are exceedingly complicated. In the case of the malarial parasite and some other protozoa, especially those which live in the blood, it is necessary for the parasite in its various stages to live in an insect host before it is mature and ready to infect man or animal again. The cycle involving sexual reproduction usually takes place in one host (the definitive host) while the asexual development occurs in another (intermediate host). This phenomenon of the developmental cycles is spoken of as *alternation of generations*. In the case of the malarial parasite the definitive host is a certain kind of mosquito, while the intermediate host is man. In the trypanosomes the invertebrate hosts may be tsetse flies and other insects, the host depending on the species of parasite.

CLASS SPOROZOA; THE PLASMODIA

Malaria. Malaria is found in all parts of the world having tropical or temperate climates and in some places it has made human habitation impossible, as in Panama a century ago and in the Pontine marshes in Italy for many centuries. Recent campaigns against malaria, however, have redeemed many such localities. With the development of DDT and more effective therapeutic drugs there is the hope that malaria may be eliminated from the United States and other parts of the world. Nevertheless it still remains one of the most widespread of the communicable diseases and is recognized as of prime importance by the World Health Organization (WHO).

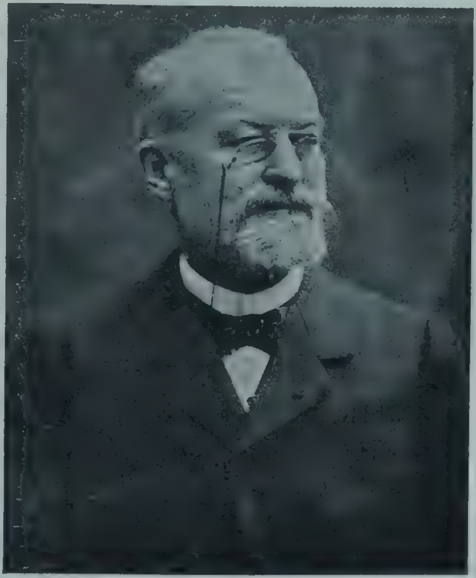

Fig. 402. Alphonse Laveran.

The malarial parasite is an extremely interesting one and has been the subject of a great deal of study. It was discovered by Laveran, a French military surgeon in Algiers, in 1881 and his discovery has affected the lives of many millions of people and the destiny of nations (Fig. 402).

Ronald Ross, in 1898, during investigations of bird malaria, which is common, discovered mosquito transmission (by a *Culex* mosquito), while Grassi and Bignami discovered, in the same year, that human malaria is transmitted by *Anopheles* mosquitoes.

The human parasites belong to the class Sporozoa, genus *Plas-*

modium. There are four species: *Plasmodium vivax, P. ovale, P. malariae* and *P. falciparum*. Parasites are also found causing malaria of birds, lizards, bats and monkeys. These are not known to infect human beings. A common bird parasite is *Plasmodium cathemerium; P. knowlesi* is found in monkeys. These are of great importance in experimental studies.[3, 4] All have similar life cycles.

Human Cycle (Schizogony or Asexual Generation). The infected malarial mosquito, during its bite, conveys the parasites into the blood of its victim with its saliva. The parasite in this stage is called a *sporozoite*. For many years it was thought that the sporozoites attack red blood corpuscles and begin to grow within them. On the contrary, recent experimental work with bird, monkey and human malaria has demonstrated that the sporozoites enter the cells lining the capillaries of the internal organs, grow and multiply.* After about a week stages develop that attack the red blood corpuscles. This important discovery[5] helps to explain why quinine is not effective during the initial stages of the disease.

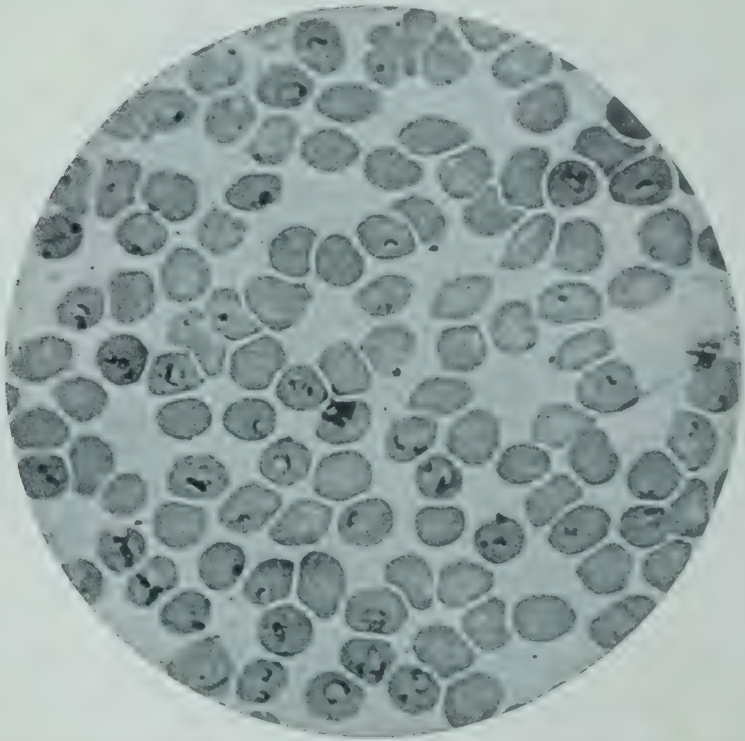

Fig. 403. Smear of blood from malaria patient showing numerous ringlike trophozoites in the red blood cells.

The parasite, inside of the corpuscle, moves in an amoeboid manner

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* Exoerythrocytic or E-E stages.

which, in *P. vivax,* is especially lively, hence the species name. Malarial pigment is formed within the parasite. The parasite is now called a *trophozoite,* at first assuming, roughly, the shape of a signet ring ("ring-stage") with the nucleus as the seal (Fig. 403). This develops into the "amoeboid stage." Each trophozoite multiplies by division, forming a number of small bodies or segments which may become arranged in a rosette. It is now known as a *schizont.* Eventually the affected corpuscle ruptures and the segments, along with pigments or metabolic products of the parasites, are liberated into the circulating blood. The patient at this time has a febrile attack often accompanied by a chill. The pigments are deposited in various tissues which eventually become deeply pigmented. Each segment is a new, active parasite and is now called a *merozoite.* As soon as it gets out into the blood, it attacks a new red corpuscle and in turn multiplies and so the asexual cycle, or schizogony, is renewed. In this way a large percentage of the red blood cells may soon contain the organisms. The patient becomes anemic and weakened due to the destruction of the red corpuscles and possibly by poisonous metabolic products of the parasites.

The successive crops of parasites mature in the blood in great groups, all the individuals of which burst out of the corpuscles at about the same time. These crops mature at different rates, two, three or four days, depending on the parasite involved. This causes the successive "chills and fever." A febrile attack in a malaria patient means that a fresh crop of parasites has entered the circulation.

After a few cycles of this sort (five to ten days), other stages of the parasite may be found. These large rounded forms that contain only one nucleus almost fill the erythrocytes in which they develop. They are the stages that will develop into the sexual forms or gametes, and are therefore called "gametocytes." Malariologists can distinguish the male and female gametocytes by their staining properties (Wright's or Giemsa's stain) and morphology. They float about in the blood stream and eventually perish unless taken up by an *Anopheles* mosquito. Their true nature then becomes apparent.

Development in the Mosquito (Sporogony or Sexual Generation). MacCallum, as a young medical student, was the first to realize the true nature of these forms while studying a similar parasite (*Halteridium*) in birds. He reported[6] concerning these gametocytes: "Some remain quiescent; others, after violent convulsions of the protoplasm, throw out long, active flagella which beat about and soon become separate, free-swimming threads, like spermatozoa. These make their way to the quiescent forms, and of the little swarm which hovers about each of these female (quiescent) forms, or macrogametes, it is seen that one and only one buries itself in the protoplasm, while the rest perish. The flagella, or microgametes, are really analogous to spermatozoa." The fertilized form of the malarial parasite is called an *oökinete*. "Sir Ronald Ross, in India, discovered that there appeared pigmented cysts in the walls of those mosquitoes which have bitten persons ill with malaria, and formed the idea that the process of fertilization and formation of a motile zygote (*oökinete*), described above must occur in the mosquito's stomach, and that the development of cysts in the walls of that organ must be due to the fact that this new, active zygote (*oökinete*) could push its way into that situation and there become encapsulated." Within these cysts or capsules (called

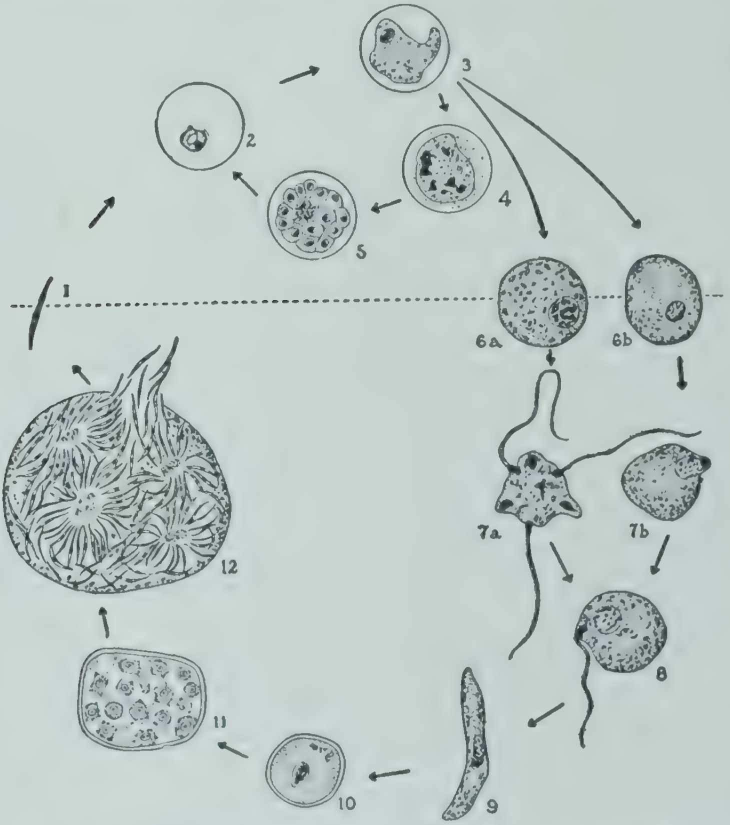

Fig. 404. Life cycle of the tertian malarial parasite, *Plasmodium vivax*. The stages above the dotted line occur in the peripheral blood of man, whereas those below are found only in the mosquito. *1*, Sporozoite; *2*, trophozoite, in red cell; *3*, full-grown schizont; *4*, schizont with chromatin in several masses; *5*, segmentation stage; *6a*, male gametocyte; *6b*, female gametocyte; *7a*, exflagellation of male gametocyte—formation of microgametes; *7b*, female gametocyte extruding chromatin from nucleus; *8*, fertilization of macrogamete by microgamete; *9*, oökinete; *10*, young oöcyst; *11*, oöcyst with many nuclei; *12*, ripe oöcyst, discharging sporozoites. The E-E stage is not shown but would occur between stages 1 and 2. (After Hegner and Cort from Hegner, Root and Augustine, "Animal Parasitology," D. Appleton-Century Co., publishers.)

oöcysts) further divisions of the fertilized parasitic cells take place and the cysts burst, liberating enormous numbers of sporozoites, some of which migrate to the salivary glands of the insects and are ready to infect man and begin the asexual cycle or schizogony again (Fig. 404). The period between the ingestion of blood by a mosquito and the time when the bite becomes infective is usually around twelve days at temperatures of 25° to 30° C.

Four Species of Malarial Parasite. One of the commonest species of the malarial parasite is *Plasmodium vivax*. Schizogony, in this species, requires forty-eight hours for completion, so that febrile attacks occur at intervals of forty-eight hours or every third day (*tertian fever*). This type of the disease is called "vivax malaria."

Plasmodium ovale causes a disease very much like malaria due to *P. vivax,* but milder. The two species are much alike.

Another species of the parasite, *P. malariae,* requires seventy-two hours for completion of the asexual generation and groups of parasites mature every fourth day producing a *quartan fever*. This form of the disease is called "malariae malaria." The amoeboid stage may assume a "band form," which extends across the erythrocyte centrally and is characteristic of this type of infection.

The fourth species of parasite is called *P. falciparum,* due to its sickle-shaped gametocytes, and matures at irregular intervals between twenty-four and forty-eight hours. Temperature and chills appear, therefore, at irregular intervals (*subtertian fever*). Only the ring stages and gametocytes are seen in the blood, other stages occurring in the capillaries. Often several rings are seen in the same red cell. Falciparum malaria which commonly occurs in the late summer and fall (*estivo-autumnal* malaria) is apt to be more severe than the other forms of malaria. A large number of the fatal cases of malaria are caused by *P. falciparum*. Relapses after apparent cure, especially in relation to changes of climate or season, are characteristic of malaria of all types.

The Malaria-transmitting Mosquito. The name of the genus of mosquitoes which transmits malaria is *Anopheles*. A number of species of this genus are capable of transmitting the disease, some being more efficient in this respect than others. Common species are *A. quadrimaculatus* (especially in the southern United States), *A. maculipennis* (on the Pacific coast) and *A. gambiae* (the most dangerous vector in tropical Africa and, a few years ago, in Brazil).[7] All have certain outstanding characteristics in common.

Only the female of any type of mosquito is equipped with biting organs. The female *Anopheles* may be differentiated from other kinds of mosquito by her position during a bite. This is indicated in Figure 405. The common, harmless *Culex* mosquito stands as shown in

Figure 405. *Anopheles* mosquitoes can usually also be recognized by the presence of silvery or gray spots on the wings and often gray bands on the legs.

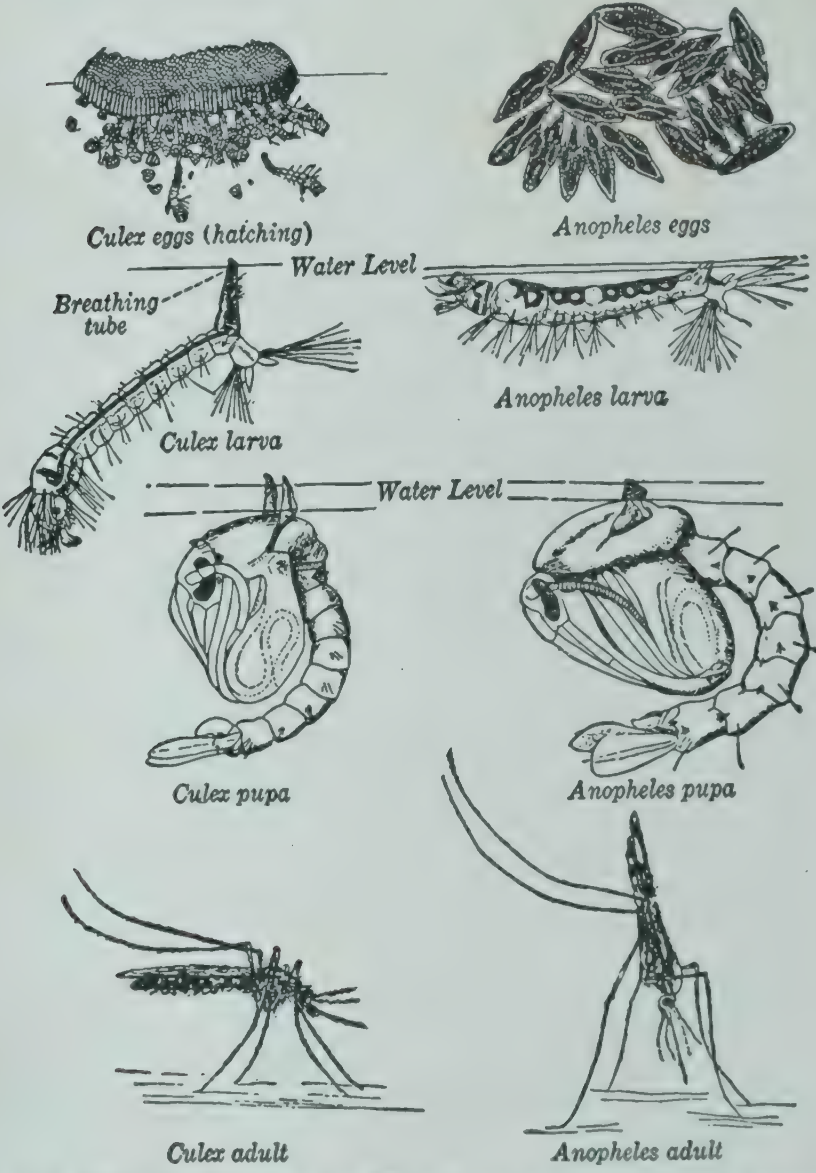
Culex eggs (hatching)
Anopheles eggs
Water Level
Breathing tube
Anopheles larva
Culex larva
Water Level
Culex pupa
Anopheles pupa
Culex adult
Anopheles adult

Fig. 405. Mosquito. Stages in the life cycle of a common house mosquito of the genus *Culex* at the left and of a malaria mosquito, *Anopheles*, at the right. (Courtesy of U. S. Bureau of Entomology.)

All mosquitoes pass through four stages of development. These are: the egg; the larva or "wiggler"; the pupa or "tumbler" and finally the fully developed insect or imago. The first three stages are aquatic.

Anopheles mosquitoes may be identified by certain characteristics of the eggs, larvae and pupae as well as by the position and markings of the adult (Fig. 405). Students who are interested are referred to books on entomology.[8]

Diagnosis of Malaria. The laboratory diagnosis of malaria is made by smearing a drop of the patient's blood evenly and thinly over a slide. It is stained with Wright's or Giemsa's stain or any of the group of polychrome stains known as Romanowsky stains which are valuable for such work. The protozoa can be seen in the blood cells and, depending on the stage of their development in the red cells, may have any of the forms shown in Figures 405 and 406. A better way of diagnosing light infections is by examining stained thick blood films

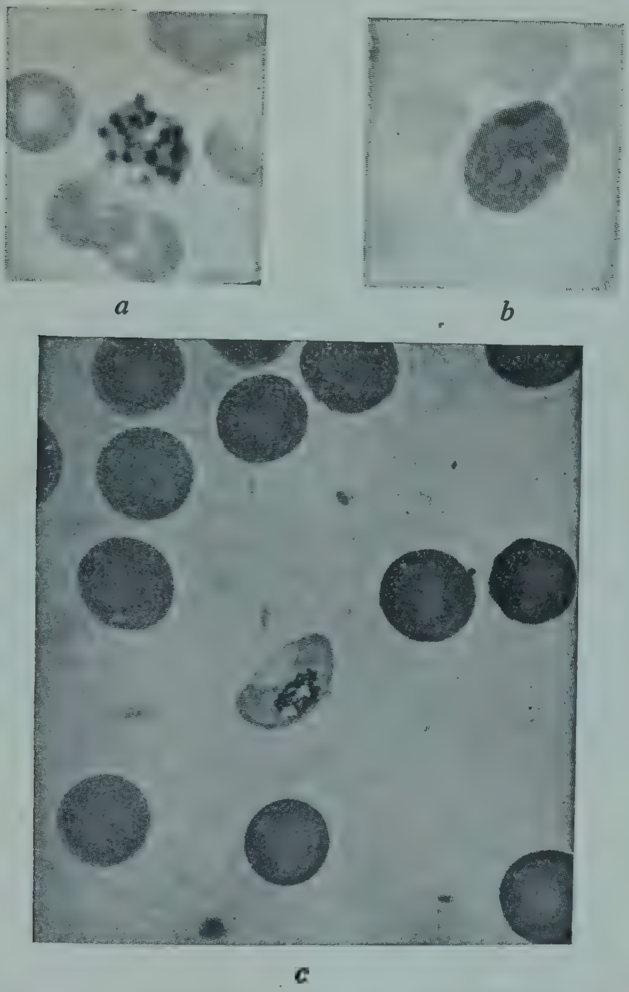

Fig. 406. *a, Plasmodium vivax* (mature schizont); *b, P. vivax* (macrogametocyte); *c, P. falciparum,* showing crescentic form. (United States Public Health Service and Army Medical School Collection, Washington, D. C.)

which tend to concentrate the parasites in a smaller area. However, it requires considerable training and experience to recognize the organisms in these preparations.[9]

CLASS SARCODINA; THE ENDAMOEBAE

The endamoebae or intestinal amoebae constitute one genus of the large group of organisms (Sarcodina) which includes the ordinary, free-living amoebae, such as *Amoeba proteus* or *Arcella vulgaris,* which every student of elementary biology has seen. Like ordinary amoebae, the endamoebae have no fixed form, but continually change their shape from round or oval to very irregular forms, with lobules and elongated portions protruding from various parts of the cell. They are about 30 microns in diameter. They move about by means of the protrusions, which are called pseudopodia (*pseudo* = like; *pod* = foot). In many respects amoebae are like the leukocytes of the blood, especially in manner of engulfing food, moving, and reactivity to various stimuli such as foreign particles. Several species of amoebae live in the human intestine. The one that is known to produce disease is called *Endamoeba histolytica* (*histo* = tissue; *lytic* = dissolving). Others are *Endamoeba coli, Endolimax nana* and *Iodamoeba bütschlii*. The last three are harmless but may be confused with *E. histolytica*.

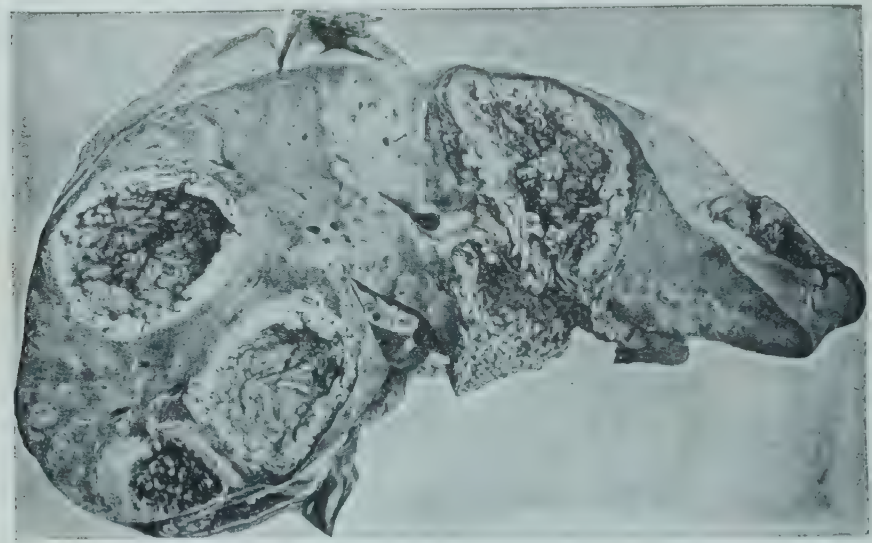

Fig. 407. Multiple amoebic abscesses of liver. (Mense's Handb. d. Tropenkrankheiten.)

Amoebiasis and Amoebic Dysentery. When human beings are infected with *Endamoeba histolytica*, the organisms establish themselves in the intestine. They attack the cells which line the colon and also ingest red blood corpuscles. They dissolve the tissues by means of a lytic enzyme. They take the corpuscles into their bodies in much the same manner as do the leukocytes. Mild or severe symptoms of amoebiasis may develop. In susceptible persons, the destructive action of these parasites may cause a severe dysentery and extensive ulceration of the large bowel. Blood is found in the stools. If allowed to extend their activities, the parasites sometimes penetrate the lining of the bowel and cause perforations of the intestinal wall. The patient may then die of peritonitis caused by the bacteria in the feces. The amoebae get into the blood vessels and are carried into the liver, lung, brain or other organs, and may excavate large cavities wherever they locate (Fig. 407).

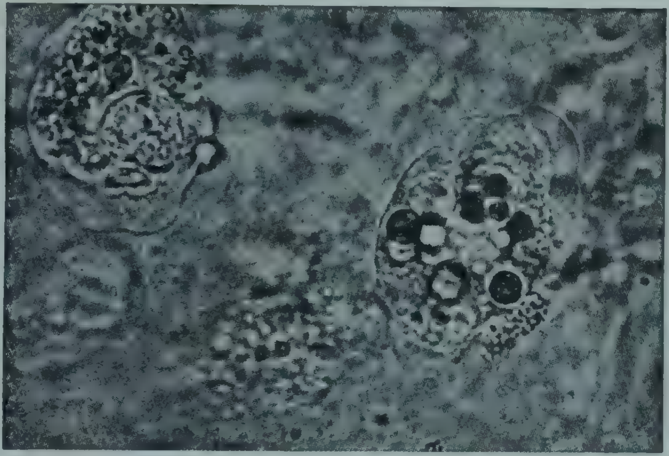

Fig. 408. *Endamoeba histolytica* (trophozoite or active form). (Army Medical School Collection, Washington, D. C.)

Transmission of E. Histolylica. During the acute diarrheic stage of the disease actively motile, relatively fragile, vegetative or trophozoite stages may be found in the loose stools (Fig. 408). If the disease is milder or becomes more chronic, the intestinal motion will be less rapid, and inactive, resistant, cyst forms will appear in the stools (Fig. 409). These cysts are often passed with the feces in large numbers. They can remain alive for days in moist feces or in damp, polluted soil or water. Anything contaminated with feces containing these cysts may therefore transmit the disease. Drought and sunlight are very unfavorable to the cysts. The trophozoites are not infectious as they are killed by gastric secretions. The cysts resist the action of the stomach but in the small intestines the digestive juices cause the organisms to escape through an opening that develops in the cyst wall. The excysted amoebae grow, multiply and may invade the mucous membranes. Acute cases are, therefore, less apt to spread the disease than carriers. In the Orient and other places where fresh sewage and human feces are used as fertilizer for truck gardening, fresh vegetables such as lettuce, celery and the like are best left alone. The mere absence of clinical dysentery from the populations furnishing such fertilizers is no safeguard against the disease, since carriers of *Endamoeba histolytica* are common. It has recently been shown that they are not at all infrequent among people in various parts of the United States. A severe outbreak of the disease occurred in Chicago in 1933 due to accidental contamination of water by faulty sewer connections.

Laboratory Diagnosis of Amoebiasis. As has been pointed out, during the acute stages the parasites appear in the feces in an active but very fragile form. If the stool is fresh they may be observed directly with the aid of a microscope. They are characterized by having progressive locomotion and may contain red blood cells. In other stages of the disease and in carriers the cysts may be found. These are about 15 microns in diameter. The cysts of *Endamoeba histolytica* are characterized by the presence of four or less nuclei. They may thus be distinguished from the cysts of a similar, but harmless, organism called *Endamoeba coli*. The cysts of the latter are usually larger than those of the former and are further distinguished by the presence of more than four (usually eight) nuclei (Fig. 410). A weak iodine solution may be used to stain the nuclei in the cysts. *Endamoeba coli* is often found in the stools of normal people. Although it is not considered to be pathogenic, its presence does indicate fecal contamination of food and water.

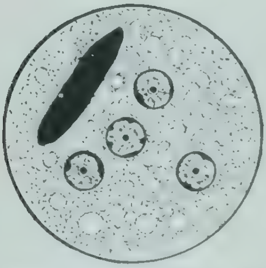

Fig. 409. *Endamoeba histolytica* (cyst or dormant form). (From Hegner, Root and Augustine, "Animal Parasitology," D. Appleton-Century Co., publishers.)

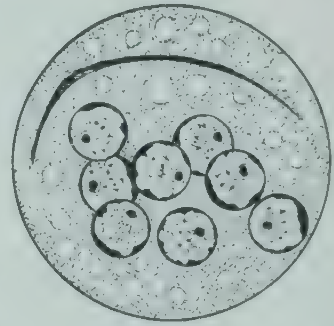

Fig. 410. *Endamoeba coli* (cyst). (From Hegner, Root and Augustine, "Animal Parasitology," D. Appleton-Century Co., publishers.)

Other aids in the diagnosis of the intestinal amoebae are staining of the specimen with iron-hematoxylin, cultivation of the parasites on B & D medium,[10, *] and concentration of the cysts by the zinc sulfate procedure. The polyvynyl alcohol fixative technique may be used to

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[*] Desiccated "Entamoeba Medium," ready for use, may be obtained from the Difco Laboratories, Detroit, Michigan.

preserve the fragile trophozoites for subsequent diagnosis by staining.[11] Promising results are being obtained with complement fixation tests especially for amoebic infection of the liver. Emetine, arsenic and iodine compounds are old standbys for the treatment of amoebiasis. Recently a new antimalarial drug, chloroquine, was found effective against hepatic amoebiasis.

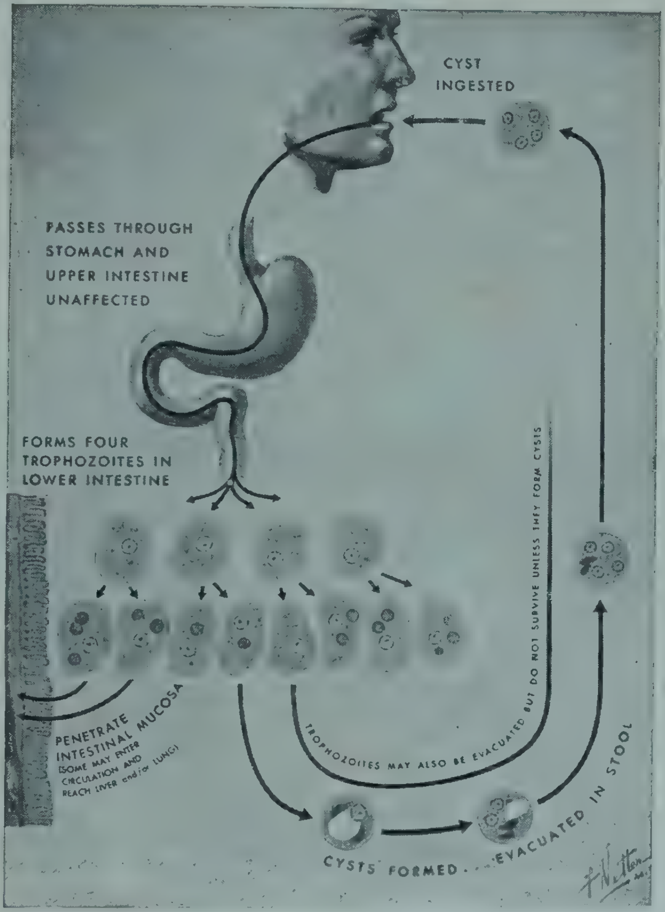
CYST INGESTED
PASSES THROUGH STOMACH AND UPPER INTESTINE UNAFFECTED
FORMS FOUR TROPHOZOITES IN LOWER INTESTINE
PENETRATE INTESTINAL MUCOSA (SOME MAY ENTER CIRCULATION AND REACH LIVER and/or LUNG)
TROPHOZOITES MAY ALSO BE EVACUATED BUT DO NOT SURVIVE UNLESS THEY FORM CYSTS
CYSTS FORMED . . . EVACUATED IN STOOL

Fig. 411. Life cycle of the *Endamoeba histolytica*. The cysts are swallowed and pass intact through the stomach and upper part of the intestine. In the lower part of the small intestine each cyst forms four trophozoites and these divide and multiply by fission. These trophozoites are motile and ingest red blood cells. Some of them penetrate the mucosa of the intestine by means of a lytic ferment which they secrete; ulcers are thus formed. Occasionally they may be carried by the blood stream to the liver where they form abscesses. Other trophozoites form cysts which pass out in the stool. These cysts, in their first phase, are uninuclear but later they form two and then four nuclei. Their large food vacuole is absorbed. If the bowels are very active, some of the trophozoites also are excreted. These may perish, or form cysts outside the body and survive. (Courtesy of Sharpe & Dohme, Inc., Phila., Pa.)

CLASS MASTIGOPHORA

Giardia lamblia. These organisms have a very characteristic appearance and are found in populations in all parts of the world (see Fig. 412). They were probably first observed by Leeuwenhoek in his own stools. In size they compare with the larger leukocytes. They live in the small intestine and fasten themselves to the cells lining it by means of a sucker-like depression, absorbing the body fluids. Like the amoebae, they can form rather resistant cysts which are disseminated by any material recently polluted with feces. The pathogenicity of *Giardia* is questionable but it may cause an irritation of the intestine sometimes resulting in diarrhea. The organisms are often found in normal people and animals. Diagnosis may be made by examination of the stools with the microscope. The cysts have a distinctive appearance (see Fig. 412).

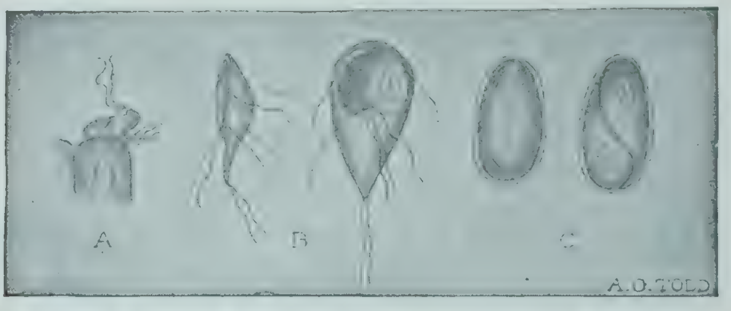

Fig. 412. *Giardia lamblia,* the most prevalent intestinal flagellate: A, Flagellate form attached to the top of intestinal epithelial cell (× 500); B, flagellate form; side and ventral views (× 1000); C, cysts, frequently seen in the feces (× 1000). (After Chandler: A following Grassi and Schewiakoff, B and C following Wenyon.)

Trichomonas hominis. These organisms are pear-shaped, flagellated protozoa which are, in general, a little smaller than the giardias (Fig. 413). They probably cause no disturbances in the intestine. Cysts are not formed. The trophozoites are quite resistant, however, and serve as a means of transmission. They are found, not infrequently, in normal persons.

Trichomonas vaginalis, a similar species, is not uncommonly found in exudates from cases of vaginitis. Apparently it is a cause of vaginitis, but is commonly found in the normal vagina, and in the prostatic secretion of man.[13]

Trichomonas foetus, closely related, infects the genitalia of cattle and produces vaginitis in cows.[14] It is of economic importance due to its relation to "delayed conception and other reproductive defects of susceptible cattle" (Andrews).

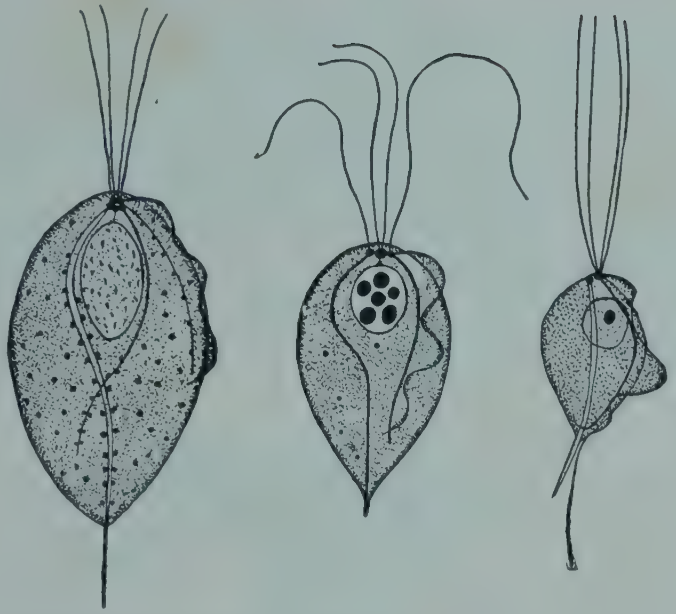

Fig. 413. *Trichomonas vaginalis, Trichomonas buccalis, Trichomonas hominis* (× 2000). (Powell.)

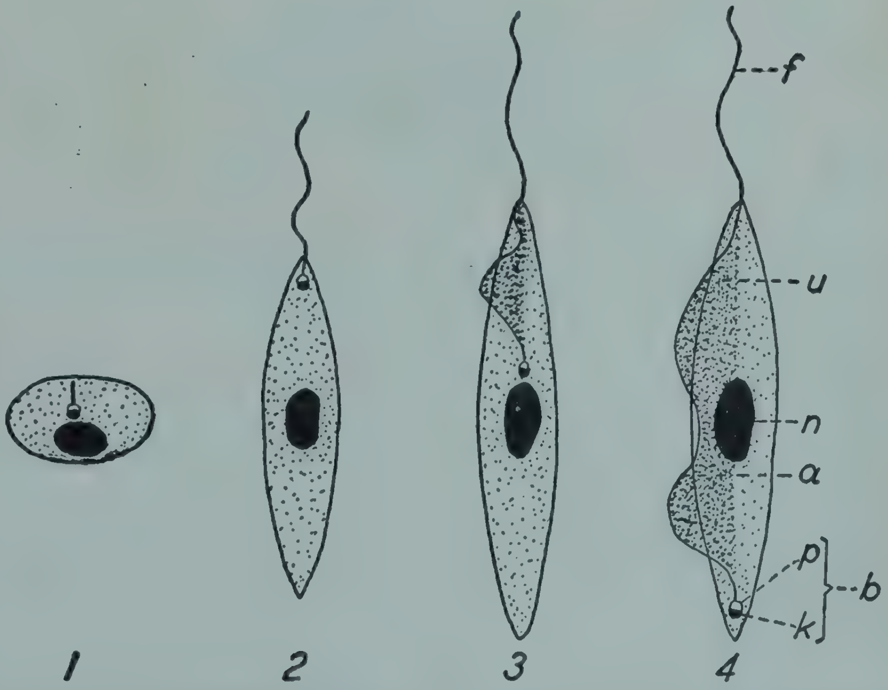

Fig. 414. Developmental stages of typical trypanosome: 1, leishmania; 2, leptomonas; 3, crithidia; 4, trypanosome. a–axoneme; b–blepharoplast; f–flagellum; k–kinetoplast; n–nucleus; p–parabasal body; u–undulating membrane. (Culbertson, "Medical Parasitology," Columbia University Press, publishers.)

The Trypanosomes. Trypanosomes are elongated, spindle-shaped organisms, extremely flexible and motile, and have the appearance shown in Figure 414; 4. They are from 15 microns to 30 microns in length and the diameter is about one-sixth of the length. In addition to a large and easily stained nucleus they have a flagellum which is attached, at the posterior end, to a complex granule called a blepharoplast and which extends to the anterior end of the body attached to the outer edge of an undulating, keel-like membrane which traverses the length of the cell. Important species in human disease are those which cause African sleeping sickness (*Trypanosoma gambiense* and *Trypanosoma rhodesiense*). A serious disease of a somewhat similar nature in South America, called Chagas' disease, is also caused by trypanosomes called *T. cruzi*.

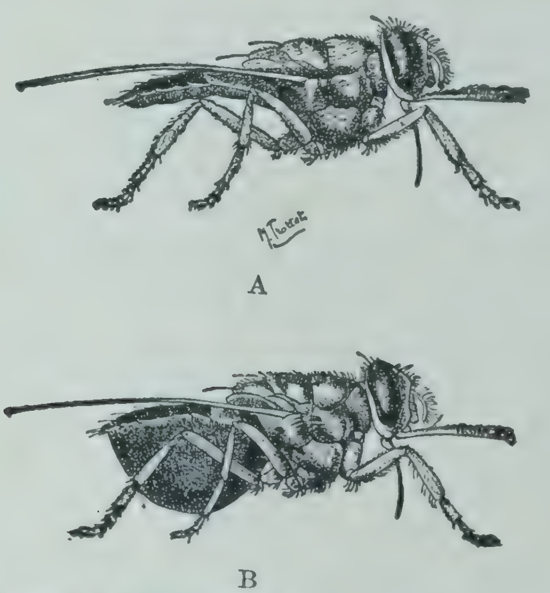

Fig. 415. Tsetse flies. *A*, Before blood meal with abdomen undistended; *B*, after engorgement with blood. (Zinsser and Bayne-Jones, "Textbook of Bacteriology," D. Appleton-Century Co., publishers.)

Many trypanosomes undergo four stages in development: the leishmania stage, the leptomonas stage, the crithidia stage, and the trypanosome stage. The first and last stages are usually spent in a vertebrate host; the other stages occur in invertebrate hosts. These four stages have not been observed to occur in all species of trypanosomes (Fig. 414). Characteristic differences of the four stages are shape, size, and development of the flagellum and membrane.

In sleeping sickness the trypanosomes live in the blood and may be readily observed when stained by Wright's or Giemsa's stain. They also occur in the lymph glands and spinal fluid. The trypanosomes of African sleeping sickness are transmitted by the bite of a fly called the *tsetse fly* (*Glossina palpalis* and *Glassina morsitans*) (Fig. 415). The trypanosomes undergo developmental changes and multiplication in the bodies of the insects. Tsetse flies occur only in Africa, and thrive especially in shaded areas along streams. Prevention of African sleeping sickness depends upon the elimination of the tsetse flies and certain wild animals which act as carriers of the trypanosomes. The untreated individual usually dies although he may live for months or years in a dull or comatose state. Treatment with arsenicals (tryparsamide) or a complex drug called germanin is effective in some cases.

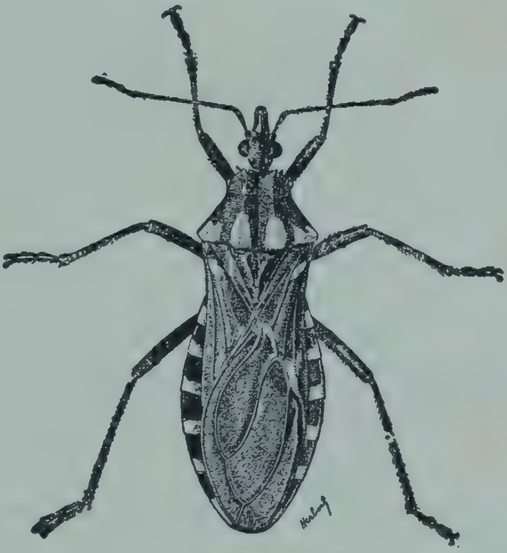

Fig. 416. *Triatoma megista,* or "barbeiro," vector of Chagas' disease. (Zinsser and Bayne-Jones, "Textbook of Bacteriology," D. Appleton-Century Co., publishers.)

Trypanosoma cruzi is transmitted by a South American insect called *Triatoma megista* or, locally, a "barbeiro" (Fig. 416). While biting, the bug passes feces that contain the infective stage of the parasite. Man becomes infected by rubbing the organism into the wound while scratching the bite. There are many animal reservoirs, and other insect vectors.

There are several trypanosome diseases of animals which cause great economic loss. *Surra* of camels and horses is one of these. Another is *dourine* of horses. Due to the fact that the latter disease is transmitted by coitus, it is often spoken of as "equine syphilis." The organism causing it is called *Trypanosoma equiperdum*. The

United States Department of Agriculture has carried on a successful campaign to eliminate dourine among wild and domesticated horses, particularly in the western part of this country. An important means of diagnosis of the disease in the laboratory is by the use of the complement fixation test. Extracts of *T. equiperdum* are used as antigen. This procedure was developed largely by Dr. H. W. Schoening, who had charge of the work.[15] It has saved farmers and ranchers millions of dollars.

The Leishmanias. These organisms in some respects resemble the trypanosomes; that is, they undergo the first two of the four stages described for trypanosomes: the leishmania stage, seen in the tissues of the vertebrate host, and the leptomonas stage found chiefly in invertebrate hosts. Morphologically the three species causing human disease (*L. donovani, L. tropica* and *L. braziliensis*) are indistinguishable. The diseases caused are different, although the ulcerations caused by *L. tropica* and *L. braziliensis* are analogous in some respects.

Leishmania donovani causes kala-azar, a disease also known as visceral leishmaniasis. It occurs chiefly in the Orient, India, Mediterranean countries and other tropical countries of the eastern hemisphere. The leishmania stage occurs in the tissues of human beings, commonly children in certain areas. Occasionally they can be found inside leukocytes in blood film stained with Wrights' stain. The organism is about 3 microns in diameter. The leptomonas stage will develop in cultures on N.N.N. medium* or in sand flies (*Phlebotomus* flies, Fig. 417).

The disease consists of a progressive and often chronic infection of the cells lining the blood vessels, especially in the spleen which becomes much enlarged. The liver, lymph glands and bone marrow also contain large numbers of the parasites. Diagnosis is often made by obtaining a bit of lymphatic tissue or bone marrow and examining stained smears of it. If bacterial contamination is prevented cultivation is not difficult and will confirm a diagnosis based on smears. Acute infections often occur and are frequently fatal unless treated with antimony compounds, especially compounds of pentavalent anti-

* *N.N.N. Medium* (Nicolle, Novy and MacNeal).

Agar Base:

Agar	14 gm.
NaCl	6 gm.
Water	900 ml.

Melt agar in the boiling salt water and make up to 1000 ml. Distribute in 4 to 5 cc. amounts in tubes. Cool to 45° C. To each tube add about 1.5 ml. of defibrinated rabbit blood. Mix, and cool with ice water in a slanting position. Saturate the cotton plugs with sterile paraffine, or use sterile stoppers. Test for sterility by incubating at 37° C. for 12–24 hours.

Inoculum is placed in the fluid at the bottom of the slants. Incubate at 22° C. for 5 to 10 days.

mony which are sometimes very effective. Recovery confers prolonged immunity. Vaccination is not effective. For many years it has been known that sand flies can harbor the organisms after biting a person with kala-azar and that this fly is prevalent in endemic areas of the disease. However, it was not until 1942 that its importance in the transmission was definitely demonstrated by infecting human volunteers through the bite of the insect.[16]

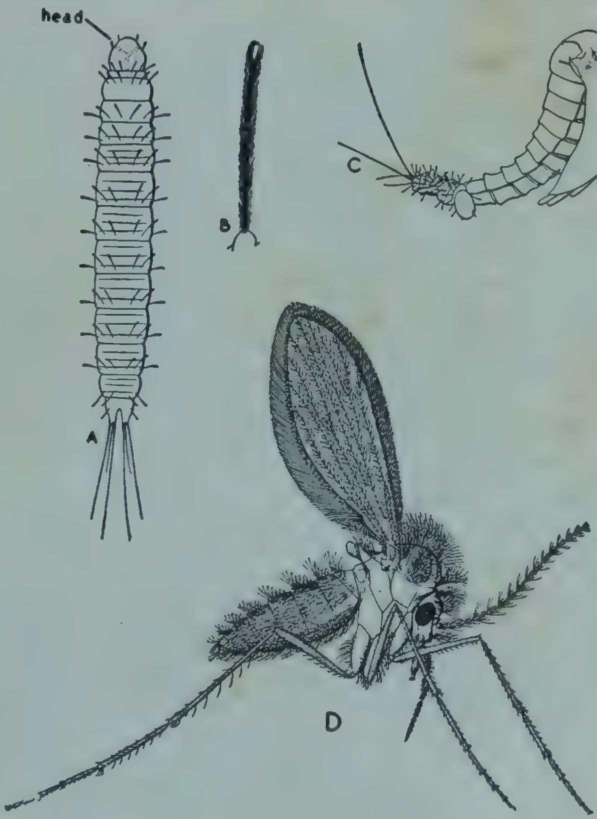

Fig. 417. Phlebotomus. *A* and *B*, The last stage larva of *P. papatasii* and one of its characteristic hairs (after Grassi); *C*, Pupa of *Phlebotomus* species (original); *D*, Adult female of *P. papatasii* (original). *A*, *C* and *D* about × 8; *B*, much enlarged. (Smart, "A Handbook for the Identification of Insects of Medical Importance," British Museum.)

Leishmania tropica, the cause of Oriental sore (Delhi boil, Aleppo boil, etc.) is very much like *L. donovani* in all respects. The lesions caused by the organism are, however, confined entirely to the skin, especially of the face and extremities but not the palms or soles. The disease is very widespread in much the same general areas as kala-azar. The same relations hold with respect to cultures and sand flies. Contact with infectious material such as actual sores, fomites, etc., may transfer the organisms from person to person. Dogs are susceptible and can carry the disease.

The sores may occur singly or in large numbers. They consist of ulcers with undermined edges, and several sores may coalesce into ulcerative areas several inches across. Secondary infection by bacteria often occurs. Healing is slow but is the rule unless treatment with antimony compounds, locally or by injection, effects an earlier cure. Scars often result from the boils (Fig. 418).

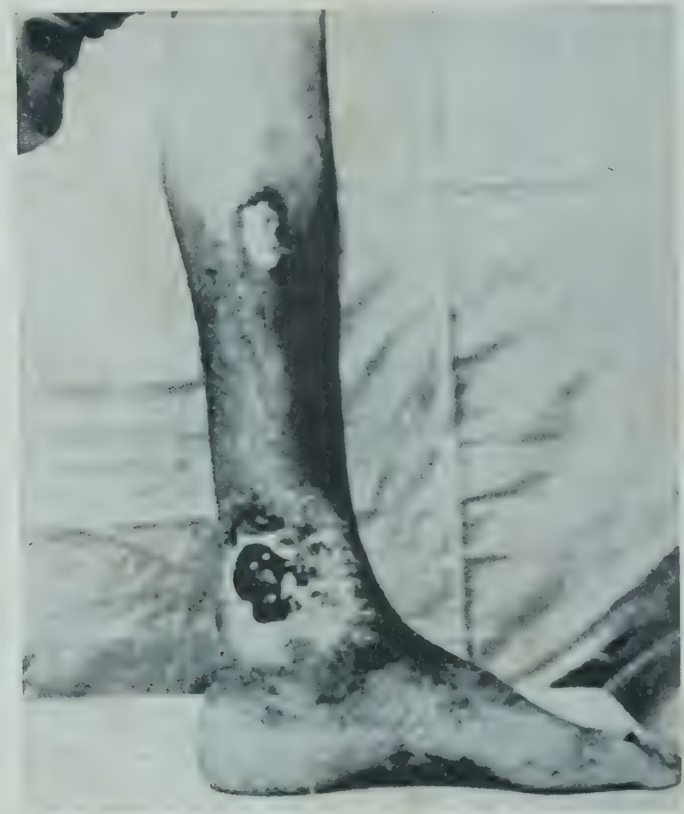

Fig. 418. Cutaneous leishmaniasis; ulcer near a malleolus; above, scar from healed ulcer. (Culbertson, "Medical Parasitology," Columbia University Press, publishers.)

Recovery results in immunity and, unlike kala-azar, vaccination by purposeful inoculation is an effective means of preventing extensive disease. It is used by natives of endemic areas for protecting children.

Diagnosis is generally not difficult since the parasites are readily seen in stained preparations made from the edges of the ulcers.

Leishmania braziliensis is so like *L. tropica* that some authorities consider it a variety of the latter. The disease caused by it (espundia, American leishmaniasis, uta, etc.) is in some respects like Oriental sore. The initial stages are similar but, whereas Oriental sore tends to heal spontaneously, espundia tends to progress. The organisms invade especially the tissues of the face, nose, mouth and tongue (Fig. 419). Enormous swelling, ulceration, and finally necrosis and destruction of tissues occurs, followed by secondary bacterial invasion and perhaps death. The organisms are seen in stained preparations from the ulcerated tissues. The mode of transmission and treatment are similar to those of *L. tropica* infection.

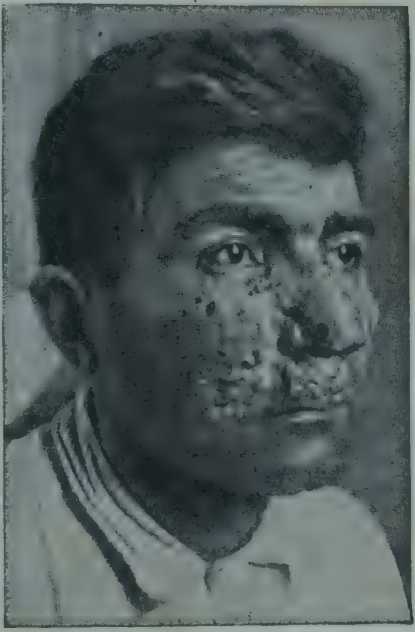

Fig. 419. Facial espundia or American leishmaniasis, showing swelling of nose, lip and adjacent tissue. (Ruge, Mühlens, and Zur Verth, "Krankheiten und Hygiene der Warmen Länder.")

CLASS CILIATA

Balantidium coli. The class Ciliata is most frequently studied through the medium of the genus *Paramecium*. Paramecia are favorite classroom material because they are harmless, large, easily obtained, have well developed organelles, and illustrate many phenomena peculiar to protozoa. Every student of biology is familiar with the "slipper animalcule" and its lively and educational antics under the microscope. Less well known is a first cousin, the intestinal parasite *Balantidium coli*. This was discovered by Malmsten in 1857 in stools of dysentery patients and has since been shown to be a pathogenic organism.

The structure of *Balantidium coli* is consistent with its relationship to *Paramecium,* but it is ovoid in shape and about 60 microns long by about 50 microns wide (Fig. 420). The life cycle involves a trophozoite stage, active and pathogenic in the intestine of man and animals, and a cyst stage during which it is dormant and resistant to conditions in the outside world. It is primarily a parasite of pigs and is common in swine. Persons who are in occupational contact with swine very frequently become infected although only a small proportion develop actual disease. Infection is transmitted in the cyst form, which develops in the intestine into the trophozoite. After varying periods cysts are found in the feces and any food, water, or other material contaminated with them may cause infection.

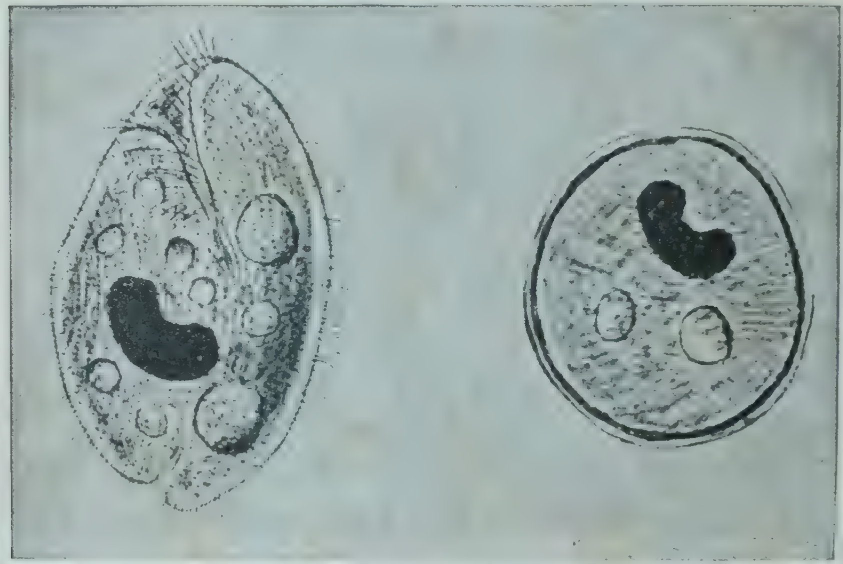

Fig. 420. *Balantidium coli.* Drawing of trophozoite (left) and cyst (right) from specimens stained by iron-haemotoxylin. × 650 app. (Lynch, "Protozoan Diseases of the Alimentary Tract," by permission of The Macmillan Co.)

The disease is analogous in some respects to amebic dysentery, with ulcers formed in the intestinal mucosa. A proteolytic enzyme is probably produced by the parasite. The resulting dysentery is usually relatively mild although bloody stools are sometimes found. The infection does not extend to other organs of the body, as does amoebiasis. Spontaneous cure is the rule. Diagnosis is made by observation of the characteristic trophozoite and cysts in the stools.

The parasite may be cultivated in B & D medium. Treatment is not usually needed but the drugs used for amoebiasis may be tried. Measures directed against infection with cysts of amoebae will also help in preventing infection with *B. coli,* but must be extended to the avoidance of materials contaminated with swine feces.

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